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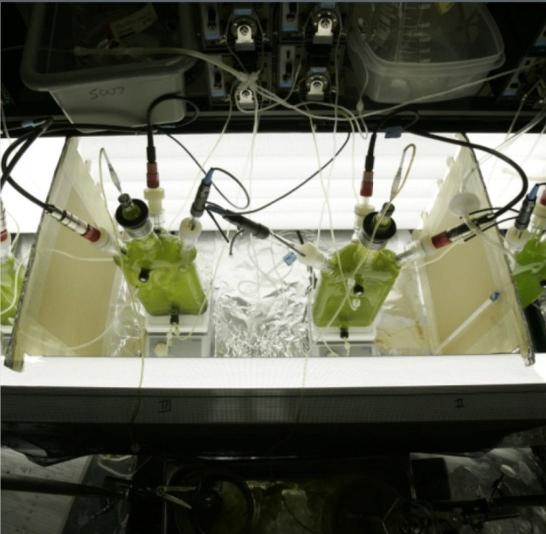

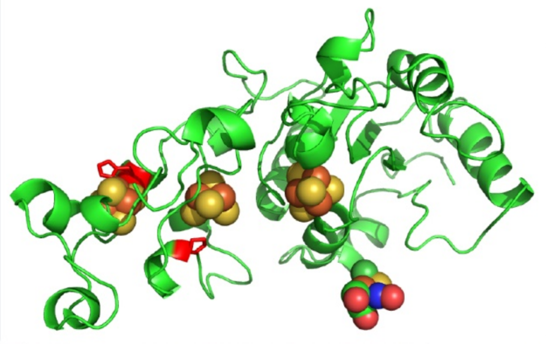

2013 Biological Hydrogen Production Workshop Summary Report

November 2013

About the Cover

(Photos from top to bottom)

Cultures of green algae producing hydrogen from water and light. Photo courtesy of National Renewable Energy Laboratory (NREL). (NREL 14579)

A model of the small subunit of a hydrogenase enzyme, showing the metal clusters as colored balls and the protein structure as green ribbons. The amino acids in red indicate substitutions that improved hydrogen evolution rates. Photo courtesy of Philip D. Weyman, J. Craig Venter Institute

Bacteria break down biomass to produce hydrogen in a fermentation system. Photo courtesy of NREL. (NREL 14576)

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2013 Biological Hydrogen Production Workshop Final Report

K. Randolph and S. Studer

Proceedings from the Biological Hydrogen Production Workshop

National Renewable Energy Laboratory

September 24-25, 2013

November 2013

Sponsored by:

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Biological Hydrogen Production Workshop

Workshop held September 24-25, 2013
Energy Systems Integration Facility
National Renewable Energy Laboratory, Golden, Colorado

Sponsored by:

U.S. Department of Energy (DOE), Energy Efficiency and Renewable Energy (EERE)
Fuel Cell Technologies Office (FCTO)

Hosted by:

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Executive Summary

Objective and Approach

The objective of the Biological Hydrogen Production Workshop was to share information and identify issues, barriers and research and development (R&D) needs for biological hydrogen production to enable hydrogen production that meets cost goals.

The workshop was divided into sessions for two topic areas: photobiological hydrogen production, and non-light driven biological hydrogen production (see Agenda, Appendix C). In photobiological hydrogen production, biological systems use light to produce energy and/or substrates for the evolution of hydrogen gas. Non-light driven hydrogen production processes are those that do not require light to function, for example fermentation of biomass or microbial fuel cell-related activities. For each topic area, a panel of experts presented on the status of the field and identified key issues and challenges to developing technologies for low-cost biological hydrogen production. These presentations can be found at http://www1.eere.energy.gov/hydrogenandfuelcells/wkshp_bio_h2_production.html. After initial presentations, a moderated panel discussion further explored the issues. The group then divided into three breakout groups, covering different sub-topics, to identify and discuss the key issues, major barriers, and high-impact R&D that would contribute to developing low-cost biological hydrogen production methods. After the breakout discussion all participants reconvened and each breakout group reported on their top issues, barriers and R&D. Each session ended with a full group discussion of the topic area, including common themes among the different breakout rooms and issues that may have been missed.

Summaries of the discussions of each topic area are included in the report, including highlights of issues, barriers and high-impact R&D as identified by workshop participants.

Summary

Across all pathways, major near-term themes included: the need to establish the necessary knowledge base and tools for the development of biological hydrogen production pathways. Across all timeframes, a major theme was the integration and demonstration of system components, technoeconomic analysis and identification of key system metrics to assess and examine production pathways. More detailed discussion can be found in Conclusions and Next Steps.

In the photobiological area, pathways are in relatively early stages of development and important areas of future study include better understanding of energy flows, target- and hypothesis-driven screens of diverse sample sets, and development or improvement of tools to enable manipulation of organisms.

The non-light driven biological hydrogen production pathways are further along in development, which is reflected in the areas of study identified for these technologies, which include scale-up and reactor design, tools to manipulate strains, and improved understanding of metabolic and energy flows.

Workshop Objectives and Organization

The Biological Hydrogen Production Workshop was held at the National Renewable Energy Laboratory (NREL) September 24-25, 2013, and included 29 participants representing academia, government and national laboratories with expertise in relevant fields. The objective of the workshop was to share information and identify issues, barriers, and research and development (R&D) needs for biological hydrogen production to enable hydrogen production that meets Department of Energy (DOE) cost goals.

The workshop began with an introductory session that included presentations on the Fuel Cell Technologies Office (FCTO) portfolio, workshop objectives and goals, and a presentation on insights from technoeconomic analysis (TEA) of biological hydrogen production pathways.

Following the introductory section, the interactive sessions began. The remaining time of the workshop was divided into sessions for two topic areas: (1) photobiological hydrogen production and (2) non-light driven biological hydrogen production methods. Each session began with a panel of experts presenting on the status of the field and identifying issues and challenges to developing low-cost biological hydrogen production. All presentations can be found online at http://www1.eere.energy.gov/hydrogenandfuelcells/wkshp_bio_h2_production.html. After the presentations, a brief moderated discussion further explored the issues. This was followed by moderated breakout groups, covering the topics listed below.

Photobiological Breakout Groups – Day 1

- **Photolytic Hydrogen Production:** Hydrogen production from biological water splitting by oxygenic phototrophs (algae, cyanobacteria)
- **Photofermentative Hydrogen Production:** Hydrogen production from light-driven fermentative processes
- **Biohybrid Systems and Enzyme Engineering for Hydrogen Production:** *In vitro* biohybrid systems and enzyme engineering for solar hydrogen

Non-Light Driven Biological Breakout Groups – Day 2

- **Fermentative Hydrogen Production:** Hydrogen production from fermentation of biomass resources
- **Hydrogen Production by MxCs:** Hydrogen production from microbial fuel cell-based technologies
- **Genetic and Metabolic Engineering for Hydrogen Production:** Genetic and (non-photosynthetic) metabolic pathway engineering for biological hydrogen production

Each breakout session was asked to generate answers to the same four questions:

- What issues need to be addressed to develop low-cost biological hydrogen production methods?
- What are the major barriers to developing low-cost biological hydrogen production?
- What R&D activities are needed to achieve efficient, low-cost biological hydrogen production, in the near-(now-2020), mid-(2020-2025), and long-term (2025 and later)?
- What are the key near-term activities for impact on production issues and barriers?

Participants were given stickers to use to vote on topics as follows:

- Which of the **barriers**, if resolved, would have the biggest impact on enabling low cost biological hydrogen production?
    - Each participant voted for up to five different topics. For topics that received votes, the total number of votes is listed in the tables in blue.
- Which of these **R&D** activities would have the biggest impact on enabling low cost biological hydrogen production?

- Each participant voted for up to five different topics. For topics that received votes, the total number of votes is listed in the tables in green.

- What is the top R&D need in the **near-term**?
  - Each participant voted for one near-term topic. For topics that received votes, the total number of votes is listed in the tables in red.

For the “pick your top five” voting rounds, individual participants could not vote more than once for the same card. The barriers and R&D topics that received votes are found in tables in the breakout discussion sections of this report. These tables have been edited slightly to clarify some of the topics. **The full tables, with all topics generated by the breakout groups can be found in Appendix B, with minimal editing beyond correcting spelling and clarifying abbreviations.** After the breakout discussion all participants met again and each breakout group reported on their top issues, barriers and R&D.

Each day ended with a full group discussion of the topic area, including common themes among the different breakout rooms, issues that may have been missed, and deeper discussion of some of the issues identified by the participants.

Introductory Session

In the introductory session (see Appendix C for Agenda), **Dr. Sara Dillich**, Acting Hydrogen Production and Delivery Team Lead in the U.S. Department of Energy (DOE) Fuel Cell Technologies Office (FCTO) welcomed participants and gave an overview of the Office and some of the goals of the workshop (see Figure 1). To get from the current status to the ultimate DOE goals, more information is needed, including: identification of relevant system metrics to be able to evaluate and compare research areas, theoretical limits and practical barriers, areas of synergy where fundamental or engineering R&D is needed or can help advance applied R&D, and identification of short- and long-term strategies for R&D trajectories.

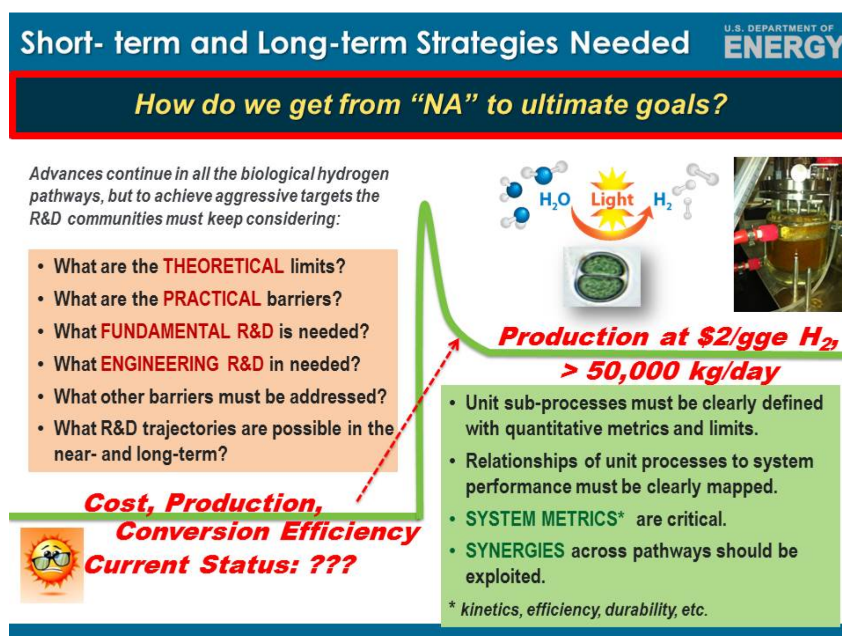

Figure 1: Identification of current status, relevant system metrics, and R&D strategies are needed in order to enable applied research into biological hydrogen production pathways, assess the potential viability of different proposed technologies, and determine key barriers and other critical areas of research focus.

Mr. Keith Wipke, Fuel Cell & Hydrogen Technologies Program Manager, NREL, and **Dr. Richard Greene**, Director, Biosciences Center, NREL, presented "*The Hydrogen Program at NREL: A Brief Overview*," describing NREL's activities in both renewable hydrogen technologies and in biosciences, with a focus on the importance of integrating different fields. **Dr. Katie Randolph**, Technology Development Manager, FCTO, DOE, and **Dr. Sarah Studer**, Energy Efficiency and Renewable Energy Postdoctoral Fellow, FCTO, DOE, served as facilitators, providing logistics for the meeting, introducing speakers and organizing breakout sessions.

Mr. Brian James (Strategic Analysis, Inc.) presented "*Techno-economic Boundary Analysis of Biological Pathways to Hydrogen Production (2009)*," speaking about a past technoeconomic boundary analysis of biological hydrogen production pathways, discussing both the process of doing the analysis and some of the results that could be relevant in looking at developing biological systems that can produce hydrogen at low cost. This study, completed in 2009, examined three different system models: photobiological reactor systems, dark fermentation systems and Microbial Electrolysis Cell (MEC) systems, and integrated systems (the report can be found at http://www1.eere.energy.gov/hydrogenandfuelcells/pdfs/46674.pdf).

Mr. James described the steps involved in the technoeconomic analysis (TEA) process, highlighting important lessons. The analysis started by assembling a strong technical team. They provided collaborative, interactive input to the process of identifying key attributes, modeling systems and performing cost analysis. Full documentation of key attributes, assumptions and pathway models was important while doing the analysis to improve understanding and achieve consistency between the pathways and for later use when the analysis was published. The process was iterative, with the inputs and assumptions of early steps being reevaluated based on the output of later steps, improving the results. Once completed, sensitivity analysis, such as tornado charts, can be used to determine the parameters that have the largest effect on the cost.

Through the process of building and validating the models and using sensitivity analysis once the models were completed, a number of issues that affect costs were identified. Modeling reactor systems helped to identify a number of issues that may be significant challenges in large-scale, long-term bioreactors that are not apparent in bench-scale experiments due to the small scale or controlled conditions. These include mixing, pH control, temperature control, and nutrient costs. An important factor for bioreactor systems was determining the appropriate module size, as modules that are too small would have high balance of labor, balance of plant and other operations costs but don't produce enough hydrogen to be economical. For photolytic systems, the co-production of hydrogen and oxygen raises safety issues and requires purification systems that significantly affect the net production amount and costs. In some systems, waste products were identified that could be sold and used as valuable sources of revenue.

Photobiological Hydrogen Production

Photobiological Hydrogen Production Presentations

Dr. Matt Posewitz (Colorado School of Mines), "*Renewable Hydrogen Production from Biological Systems*." Dr. Posewitz, the panel leader, began with an overview of photobiological hydrogen production methods. He noted that in the last few years there have been significant advances in the field, and that the paths forward are clearer than before. There are several potential hydrogen pathways found in various photosynthetic microorganisms, those that utilize sunlight for energy. Hydrogen production can result from the Photosystem II (PSII)/Photosystem I (PSI) pathway, from the PSI/nonphotochemical plastoquinone (PQ) pathway, and/or from the "dark" fermentation of starches and sugars from CO_2 fixation. Hydrogen uptake also occurs through the oxyhydrogen and photoreduction reactions involved in CO_2 assimilation. Two types of enzymes, hydrogenases and nitrogenases, produce hydrogen.

Hydrogenase, an enzyme that combines electrons and protons to produce the H_2 molecule, has been found in phototrophic microbes. The diversity of known hydrogenases has increased in recent years. [NiFe]-hydrogenases are reversibly inhibited by oxygen and fall into four groups: (1) membrane-bound uptake enzymes, (2) cyanobacterial uptake and oxygen tolerant hydrogen sensing enzymes, (3) bidirectional, NAD(P)H-linked enzymes, and (4) ferredoxin-linked hydrogen production enzymes. [FeFe] hydrogenases are capable of very high turnover over a thousand times per second, are typically irreversibly inhibited by oxygen (though differences in oxygen tolerance have been reported), and are often ferredoxin-linked, while Hnd hydrogenases are a group of multimeric hydrogenases that are linked to NAD(P)(H).

Cyanobacteria have so far only been found to have [NiFe] hydrogenases that use NAD(P)H as the electron donor, and typically produce hydrogen during the dark cycle. To date, eukaryotic algae have only been found to have [FeFe] hydrogenases, usually with two hydrogenases.

Nitrogenase enzymes also produce hydrogen, combining N_2, protons and electrons to produce NH_3 and H_2. Different enzymes have different reaction stoichiometries, but all require 2 ATP per electron. While the use of ATP makes this reaction more energetically costly, it gives the reaction a thermodynamic driving force, making it essentially irreversible. Nitrogenases are oxygen sensitive, and some species have developed methods to deal with the sensitivity, such as spatial separation in heterocysts or temporal separation. Mutants with increased hydrogen production have been reported.

Dr. Posewitz also described some recent advances in the field. Dr. Tasios Melis and colleagues developed a method for prolonged hydrogen production using nutrient stress to balance the photosynthetic oxygen production with the respiratory oxygen utilization. This has provided a way to demonstrate improvements in hydrogen production, including the enhancement of hydrogen production by photosynthetic antenna mutants with improved light utilization. Sustained biophotolysis has been demonstrated in the cyanobacteria *Cyanothece* using nitrogenase catalyzed hydrogen production. The rates of hydrogen production were up to 400 µmole/hr·mg chlorophyll, with sustained coproduction of hydrogen and oxygen. Other researchers have worked with the interplay of photosynthesis and starch for hydrogen production, demonstrating that starch hyperaccumulation strains produced high hydrogen yields. Screening methods that use heterologous expression and screening of hydrogenases in alternate hosts and reverse genetics have also been used. The alga *Chlamydomonas reinhardtii* has long been studied for hydrogen production, and has two [FeFe] hydrogenases that can participate in either

photobiological or fermentative hydrogen production; knock-out lines are now available. Genetic analysis is also yielding information, such as the evolutionary relationships of different versions of the HydA hydrogenase, indicating a common ancestor. Dr. Posewitz noted that ultimately solutions will need to involve docking the hydrogenases into cellular metabolism, which has lots of players to consider in terms of where the electrons are going and where the ATP demands are.

Dr. Jake McKinlay (University of Indiana), "*H_2 Production by Anoxygenic Purple Nonsulfur Bacteria*." Dr. McKinlay discussed photosynthetic hydrogen production by anoxygenic purple non-sulfur (PNS) bacteria. These bacteria use light and organic compounds for energy and electrons, and, unlike photolytic organisms, do not split water and therefore do not produce oxygen as a product of photosynthesis. These bacteria produce hydrogen using nitrogenases, with the electrons and protons coming from the metabolism of organic compounds, and the ATP required for the nitrogenase reaction being produced through photosynthesis. Hydrogen gas is an obligate product of the reaction and always produced. When fixing nitrogen, the reaction is $N_2 + 8H^+ + 8e^- + 16ATP = H_2 + 2\ NH_4^+$. In the absence of a N_2 molecule, the other reactants can produce 4 H_2 molecules. Currently, systems with growing liquid cultures can produce yields of 10%-25% of the theoretical maximum. PNS bacteria can be immobilized and put into a non-growing state, and under these conditions can produce 40%-91% of the theoretical maximum. Sustained production has been demonstrated for over 4000 hours, with no indication that production rates would drop if the experiment continued longer. Photosynthetic efficiencies are generally 1%-2%, with one report of 6%.

Dr. McKinlay described several barriers and potential methods to address them. Nitrogen fixation is energetically expensive to run so cells have developed methods to suppress the nitrogenase reaction if NH_4^+ is present, which is a particular challenge if using waste streams for feedstocks as they tend to contain large amounts of NH_4^+.This can be bypassed through regulatory changes, with at least two different mutations (*nifA** and DraT) having been shown to overcome the inhibition. Competing metabolic pathways can reduce efficiencies and yields, and efforts are underway to reduce these, such as the identification and elimination of the Calvin cycle. Biosynthesis for cell growth competes with hydrogen production, but improved use and understanding of non-growing cells has resulted in cells with high hydrogen yields. Limited light penetration into the cultures, which means that cells deeper in a culture receive little to no light while the top layer of cells receive more than they can process, can be addressed at both the organismal level through pigment mutants, and at the system level through novel bioreactor designs. The integration of these systems with "waste" feedstock streams is a challenge, and may be addressed by consolidating fermentation systems with the photosynthetic systems.

Research needs suggested by Dr. McKinlay include system-wide approaches to understanding genetic and metabolic factors involved in hydrogen production, particularly hydrogen production rate, as efforts to shift pathways have often resulted in yield increase but not rate increase (and sometimes showed rate decrease); examination of the physiology of non-exponential phase cells; biological and physical solutions to address light limitation with scale up; design principles of light harvesting units; and interactions with groups that can identify and implement physical solutions (e.g., light conducting plastics for use in photobioreactors).

Dr. John Peters (Montana State University) "*Hydrogenases and Barriers for Biotechnological Hydrogen Production Technologies*." Dr. Peters gave an overview of issues related to hydrogenase enzymes. He started by noting that hydrogenases are a profound case of convergent evolution – while both [FeFe] and [NiFe] hydrogenases catalyze the reaction of $2e^- + 2H^+ \leftrightarrow H_2$, they have different evolutionary origins. [FeFe] hydrogenases are found in bacteria and lower eukaryotes, and are most closely related to the protein Nar1, which is likely involved in FeS cluster repair and biosynthesis. [NiFe] hydrogenases on the other hand are found in bacteria (including cyanobacteria) and archaea, and are related to Respiratory Complex 1. The active site metal clusters of both types of hydrogenases are sensitive to oxygen.

Dr. Peters discussed a number of barriers. Oxygen inactivation of the enzymes is caused by degradation of the active site cluster. Dr. Peters' lab has worked in this area for a long time but the biochemical difficulties of oxygen sensitivity has limited study, and the pathways and mechanisms of degradation are not fully understood. When the [FeFe] hydrogenase H cluster is degraded, the 2Fe metal subcluster is lost first, followed by the [4Fe-4S] cluster. *In vitro*, the activity of enzyme that has lost these metals can be restored through adding the metal clusters followed by activation, while within cells, there are mechanisms to reconstitute the enzymes. Another barrier is oxygen sensitivity, based on the access of oxygen to the active site. Work on understanding this access is ongoing, and recently a unique FeS cluster involved in oxygen tolerance in [NiFe] hydrogenases has been identified, but it's not clear if this is a tractable problem. Active site biosynthesis and cluster maturation is an area of critical information for enzyme engineering and heterologous expression. Other challenges are defining integration of hydrogenases into metabolism and electron transfer pathways, and identifying best model organisms and opportunities for improvement.

Dr. Eric Hegg (Michigan State University), "*H_2 Production by Oxygenic Phototrophs*." Dr. Hegg presented on hydrogen production oxygenic phototrophs – prokaryotic cyanobacteria and eukaryotic green algae. Dr. Hegg began by noting that these organisms split water, which produces lots of electrons with many potential fates – for hydrogen production, the goal would be to push those electrons to hydrogen evolution. He then identified major technical and biological challenges to hydrogen photoproduction. Technical challenges include dealing with the mixture of hydrogen and oxygen that is co-produced by water photolysis and must be separated and stored, and issues of overall reactor design and CO_2 supplementation, specifically how to get the CO_2 to cells while they are actively growing. Biological challenges were the oxygen sensitivity of the hydrogen-forming enzymes, and the poor efficiency of hydrogen production. Causes of the low efficiency include poor heterologous expression of hydrogen-forming enzymes, low quantum yields, especially under high light, and competition for reducing equivalents and poor electron coupling. Dr. Hegg focused on the electron transfer and oxygen sensitivity barriers during his presentation.

Improving electron transfer could involve several different approaches: (1) Eliminate or down-regulate pathways that compete for electrons, such as the production of organic acids and the formation of NADPH and carbon fixation. This strategy depends on having good tools for genetic manipulation and an understanding of the metabolic pathways. (2) Identify the best electron transfer partner among the various ferredoxins and cytochromes. (3) Engineer improved electron coupling, for example by mutating the docking site for enhanced binding, fusing the hydrogenase to ferredoxin, fusing hydrogenase directly to PSI, or localizing the interacting partners to a synthetic protein scaffold where they are more likely to interact and send the electrons in the right direction.

Three methods to overcome oxygen sensitivity were discussed: utilizing non-oxygenic photosynthesis, engineering enzymes to be less oxygen sensitive, and separating hydrogen and oxygen biosynthesis. Non-oxygenic photosynthesis could be done using purple bacteria such as *Rhodobacter sphaeroides*, though this method would need electrons as discussed by Dr. McKinlay; using selective light that preferentially activates PSII, though it is not clear how well this would work on a large industrial scale; or using a method like sulfur deprivation, which results in no net production of oxygen. Enzymes could be engineered to be less oxygen sensitive by inhibiting diffusion of oxygen or altering the redox potentials, which might be difficult due to thermodynamics. Hydrogen and oxygen biosynthesis could be addressed by temporal separation of the production cycles, for example by having hydrogen produced at night by fermenting products of photosynthesis so that hydrogen is produced when the oxygen-producing photosynthesis reactions are not working, or through spatial separation. Heterocyst-forming cyanobacteria are an example of spatial separation, as in the expression of [FeFe]-hydrogenase in *Anabaena* sp.

PCC 7120. Mutations can increase the frequency of heterocyst compartments. Other types of compartments, such as carboxysomes, could be considered.

Identifying new organisms was an area Dr. Hegg also discussed. Two recently discovered organisms were described. *Cyanothece* sp. ATCC 51142 is a cyanobacterial strain that produces hydrogen from nitrogenase at rates of up to 465 umol H_2/mg chlorophyll/hr. Simultaneous light-driven hydrogen and oxygen production has been demonstrated for over 100 hours in the presence of CO_2, though the mechanism is not yet fully understood. *Volvox carteri* is a species of multicellular green algae, the first multicellular eukaryote discovered to exhibit hydrogen production, and while the production is low there is the potential for higher levels. *V. carteri* has differentiated cells, with a large central cell with smaller cells on its surface; cell differentiation means there are already multiple compartments, which could be useful in addressing oxygen tolerance.

Dr. Lisa Utschig (Argonne National Laboratory), "*Utilizing Nature's Designs for Solar Energy Conversion*." Dr. Utschig concluded the panel presentations by discussing biohybrid/cell-free systems as ways to utilize nature's designs for solar energy conversion. Dr. Utschig began by noting that fundamental studies of photosynthesis allow us to create new materials that capture, convert and store sunlight. Artificial systems for hydrogen photocatalysis can be multi-molecular or supra-molecular (with linked molecular modules), but have a number of limitations: large solvent and molecular dependencies, diffusion requirements, lifetime limitations, uncontrolled back-reactions, the noble metal content of most photosystems, and organic solvent/high proton requirements. Dr. Utschig discussed one biohybrid model that combines artificial systems with biological molecules, using the biological reaction center proteins to drive the abiotic catalysis of hydrogen production. The fundamental challenges of the system are efficient coupling of photons to fuels, creating sustainable systems, and developing materials with cheap, scalable methods for processing.

One such biohybrid system is a PSI-Pt nanoparticle hybrid system developed at ANL, in which the photosystem readily self-assembles with Pt-nanoparticles and is not covalently bonded. This system has the best PSI-Pt photo-hydrogen evolution to date and outperforms currently reported rates for photosensitizer-catalyst systems. Study showed that the Pt-nanoparticle mimics the native acceptor protein which increases the electron transfer flow and aids in high rates, and that a direct wire to the co-factor is not needed. During the question-and-answer session, it was clarified that the PSI is isolated from cyanobacteria and is a trimer, and that with a turnover of 20,000 times per minute, the limiting factor is using up the electron donor.

An alternative system uses a transition metal catalyst, cobaloxime, which is inexpensive and earth abundant as well as oxygen tolerant, instead of expensive and oxygen-sensitive Pt. Cobaloxime, a Ni-diphosphine catalyst, also enables tunability of the system. The cobaloxime is connected to a reaction center protein, which nature has optimized for solar capture and conversion. This system has demonstrated rapid light-induced hydrogen production, out-performing completely artificial systems, in completely aqueous solutions. Study of the system showed protein directed delivery of the catalyst to PSI, and the protein was shown to stabilize the catalyst, which displayed unprecedented chemistry for a Ni-diphosphine catalyst, and indicates a strategy for self-repair.

Photobiological Hydrogen Production Panel Discussion

The panel presentations were followed by a general discussion.

An early topic discussed was the *Cyanothece* system introduced by Dr. Posewitz. Dr. Alex Believ, whose lab reported the results, was a participant and filled in details in response to questions. *Cyanothece* was shown to produce hydrogen and oxygen simultaneously for extended periods of time in the presence of CO_2, which is

required to maximize the reductant flux to the nitrogenase. In the presence of CO_2 there is cycling between hydrogen production and oxygen production, which Dr. Beliav said may be related to a circadian regulation, providing temporal separation. In the absence of CO_2 there is no separation and while the cells can be maintained alive in this environment, the proteins are degraded. Based on mass balance analysis showing that the hydrogen production is above the maximum yield from glycogen metabolism, Dr. Beliav has concluded that the hydrogen is produced from both water splitting and carbohydrate metabolism, which is why the DCMU inhibitor is able to initially inhibit oxygen but not hydrogen production. Both PSII and PSI activities are needed: PSII for water splitting, and PSI to generate the ATP for the nitrogenase. It was suggested by other participants that this was similar to the sulfur deprivation system, where hydrogen comes from both sources. It was confirmed that hydrogen production takes place in the dark.

Another topic that came up repeatedly was systems that produce hydrogen from carbohydrates generated through photosynthesis, which could both allow the temporal separation discussed by Dr. Hegg and address some of the other hydrogen production rate and yield limitations. One issue with this method is the added energy cost of using carbohydrate intermediates. The theoretical maximum amount of hydrogen production would depend on what pathways were used – for example, glycolysis plus the tricarboxylic acid (TCA) cycle versus the pentose phosphate pathway. Generating carbohydrates during the day and then generating hydrogen at night will generally be somewhat less efficient than direct fermentation, as additional photons will be needed to generate the hydrogen from carbohydrates, and one expert noted that, in general, the less direct pathways are more complicated. Another expert noted that one of his students did a bioprospecting study for cyanobacteria that produce hydrogen, and the strain with the highest amount of hydrogen turned out to produce all the hydrogen from fermentation, raising the question of the potential advantages of a two-stage system. There was a type of two-stage system modeled in the TEA analysis described by Mr. James during the introduction, resulting in high costs that were mainly attributed to scaling limitations.

It was noted that while it has been claimed that NADPH is not effectively utilized in the cyanobacteria system, multiple researchers found that it worked well in their experiments suggesting that the conclusion may be based on specific systems and/or conditions and may not be broadly applicable. Also, while NADPH may be effective *in vitro*, it is less clear what the concentrations and effects are *in vivo*.

The advantage of having higher turnover rates for hydrogenases than the photosystems that produce the substrates was questioned. It was noted that those values are based on *in vitro* results and may be different *in vivo*, and that there may be multiple ferredoxin sources per hydrogenase.

It was suggested that to get a higher flux through PSII and PSI, photophosphorylation would need to be uncoupled as the PQ step is the rate limiting step of the electron transport. To select for such a strain, one could chose a strain with a high “overhead” for living in its environment, as it would be selected for high electron transfer requirements already. It may also be possible to manipulate the electron flux by altering light capture.

The mechanisms for hydrogenase assembly, repair and degradation, and the implications for both *in vivo* and *in vitro* activity were discussed. “Self-repair” is often cited as an advantage of biological systems, and some of the mechanisms may work in cell-free systems. In fact, because inside living cells, damaged hydrogenases may need to be protected from damage or turnover until they can be repaired, cell-free systems may not need all the assembly proteins that are required *in vivo*.

Photobiological Hydrogen Production Breakout Discussions

Photolytic Hydrogen Production

The breakout group discussed a number of issues that need to be addressed to develop low-cost photolytic hydrogen production methods, ranging from reactor system engineering to molecular biology issues. Reactor system issues included development of low-cost bioreactor designs, bubbler systems, which could potentially take advantage of the gases produced by the system, separation of hydrogen and oxygen mixtures, and hydrogen collection, purification and storage.

Most participants agreed that the oxygen sensitivity of the hydrogenase is a major issue. One related issue was using metagenomics to identify alternative enzymes (metagenomics refers to the analysis of genetic material is isolated from an environment without the need to cultivate the organisms). Identifying co-culturing methods for oxygen usage is another potential issue. Other compounds beside oxygen can have an inhibitory effect on hydrogenase activity; for example NH_3 inhibits the nitrogenase expression, and high partial pressures of hydrogen will reduce the rate of hydrogen production. Some hydrogenases are bidirectional, producing or consuming hydrogen depending on reaction conditions and hydrogen partial pressure. Others are strongly unidirectional but are uptake hydrogenases, so identifying unidirectional hydrogenases that produce hydrogen will be important in enabling low cost biological hydrogen production.

Another set of challenges involved altering cellular pathways to increase hydrogen production. Electrons, carried on various molecules that can be used as reducing agents, are needed for hydrogen production. To increase the electrons available for hydrogen production, the electron flux toward hydrogenase enzymes could be increased, perhaps though designing alternative pathways, and/or competitive pathways that also use those substrates could be down-regulated or eliminated. Many cellular activities that are needed for hydrogen production are down-regulated during the production process and ways to alter the regulation are needed. The Thauer limit is another metabolic pathway issue – currently once organic compounds are broken down to acetate there are no further metabolic pathways that yield hydrogen; this is sometimes referred to as the acetogenic limit. If this limit cannot be overcome through reengineering metabolic pathways, the acetate could potentially be used for other biofuel production pathways by the cells to produce fatty acids, or used as feedstocks for MxCs or photofermentation systems. Though not directly related to hydrogen production, maximizing the value of the other products of the system, such as the cellular biomass or the co-produced oxygen, could help to make the entire system more economically feasible.

One possible system configuration would be to build carbohydrates, specifically glycogen or starch, during the day and store the molecules until they can be converted to hydrogen by the cells at a later time. To be successful, the accumulation of glycogen/starch and the conversion steps to produce hydrogen would need to be improved.

During the report-out, the group summarized the barriers into three groups: regulation of pathways, optimizing the flow of reductant to hydrogen production while minimizing other products; enzyme inhibition, including oxygen sensitivity, and carbohydrate storage and utilization to optimize hydrogen production. Table 1 lists the barrier topics that received votes.

The regulation of pathways included barriers such as the need to maximize electron flux toward hydrogen production, linking new (oxygen tolerant) enzymes to photosynthetic pathways *in vivo*, the down-regulation of hydrogen production due to the non-dissipated proton gradient, competing pathways, and alternative electron sinks. Barriers related to optimizing hydrogen production by using carbohydrates include the need to accumulate

more glycogen and starch while accelerating the catabolic breakdown to match the diurnal cycle; and increasing the yield of carbohydrate catabolism to make CO_2 and H_2 by overcoming the Thauer fermentative barrier.

Other challenges relate to engineering: hydrogen collection and storage and the separation of hydrogen and oxygen.

Table 1. Photolytic Biological Hydrogen Production Barriers*

• Directing electron flux to H_2 production (5)
• Photosynthetic carbohydrate storage and conversion to H_2 (4)
    ○ Accumulate more glycogen and starch; together with accelerating catabolic breakdown to match diurnal cycle
• Oxygen sensitivity (4)
• Carbohydrate Conversion (3)
    ○ Increase yield of carbohydrate catabolism to make CO_2 and H_2 by overcoming the acetogenic fermentative barrier.
• Linking new (O_2 tolerant) enzymes to photosystem *in vivo* (3)
• H_2/O_2 separation (3)
• Enzyme inhibition (2)
• H_2 capture and storage (2)
• Down-regulation of H_2 production by non-dissipated proton gradient (2)
• Competing pathways (2)
• Maximizing reductant partitioning to hydrogenase/nitrogenase (1)
• Alternative electron sinks (1)

* Each participant voted for up to five different barriers. Total votes are indicated by the blue numbers. Only topics that received at least one vote are shown; in some cases the barrier topics were worded as solutions by the breakout participants.

For R&D, a large part of the discussion involved the tools and methods that are needed to reach the ultimate goals (Table 2 shows the R&D topics that received votes). These tools, if available, would enable or accelerate a larger set of activities. The near-term R&D with the most votes was the development and application of metabolic engineering and synthetic biology approaches for manipulation and optimization of reductant flux to hydrogen. Another highly rated topic was methods to be able to select for hydrogen production *in vivo* after mutagenesis and directed evolution – as one participant put it, methods to have cells "make more hydrogen or die." A selection method would be useful for sorting through strains generated by either random or targeted mutagenesis, directed evolution, or even bioprospecting. One potential selection method would be to design an aptamer system, which has been used in other systems to design circuits to sense and select for certain products.

Integrating current improvements into a single organism and identifying further barriers was another highly rated near-term R&D topic, extending into the mid-term. Other near-term R&D involved improvements to microbial systems: using directed evolution to improve electron flow from water splitting; improving the movement of electrons to hydrogenase using mutants that have competing pathways blocked and improved electron donation from NAD(P)H and/or ferredoxin; and improving the cells' ability to accumulate glycogen and starch and convert those carbohydrates to CO_2 and H_2 as completely as possible.

In the mid-term, the top R&D topics were: repurposing existing cellular compartments for hydrogen production, and hydrogen storage and milking methods. "Milking" removes hydrogen from the system to help pull unfavorable equilibriums toward the production of hydrogen. Compartments would provide spatial separation for the hydrogen production machinery, similar to how some nitrogen-fixing cyanobacteria use heterocysts for nitrogen fixation. This separation could protect the hydrogen production enzymes from inhibitory compounds like oxygen and/or concentrate the needed reactants. Compartments that could be repurposed include heterocysts and

carboxysomes. Other mid-term R&D topics were to engineer biological systems for high efficiency hydrogen production, and to demonstrate hydrogen production in non-growth cultures, which would reduce the energy and reactants directed toward the "overhead" costs of cellular growth.

In the long-term, the top R&D topic was systems integration, specifically combining the biology and engineering components. Other long-term R&D topics are gas separations, synthetic compartments for hydrogen production that can function *in vivo*, comparative analysis, and hybrid systems.

Table 2. Photolytic Biological Hydrogen Production R&D*	
Near-term	• Development and application of metabolic engineering and synthetic biology approaches for manipulation / optimization of reductant flux to hydrogen (3) • Integration and identification of further barriers (4, 2) • Improve electron flow from water splitting via directed evolution (1, 2) • More effectively move electrons to hydrogenase, mutants with competing pathways blocked, subunits for NADH, NADPH, ferredoxin donation (2) • *In vivo* hydrogen [production] selected after mutagenesis / directed evolution (3) • Accumulate glycogen / starch to convert to CO_2 and H_2 100% (1)
Mid-term	• Hydrogen storage and hydrogen milking (3) • Repurpose existing compartments for H_2 production (3) • Engineering biological systems for high efficiency-productivity of H_2 production (2) • Demonstrate H2 production in non-growth culture (1) • Integration of solutions (1)
Long-term	• System integration: biology and engineering (5) • Design/construct "synthetic hydrogenosome" *in vivo* (2) • Gas separation (2) • Comparative analysis / create hybrid systems (1)

* Each participant could vote for up to five different R&D topics across all timeframes; the total of these votes are indicated by the green numbers. Each participant could vote for one top R&D topic in the near-term; the totals of this near-term topic vote are indicated in red. Only R&D topics that received at least one vote are shown in this table.

Photofermentative Hydrogen Production

Photofermentation systems were considered to be microbial systems that produce hydrogen using light and electron sources besides water, whether those sources are organic or inorganic. The photofermentation breakout group identified a number of issues that need to be addressed to allow the technologies to meet DOE production goals. One issue was identifying a low-cost organic/electron sources as feedstocks. Waste streams, such as glycerol from biodiesel production, food processing waste or livestock waste, are a potential low-cost feedstocks, but current studies focus on small organic acids. Another option would be integrating multiple microbial systems, for example using biomass generated by photolytic algal systems. Other issues included conversion efficiency, light availability, the potential for contamination, and methods to harvest the hydrogen. Some of these issues could be related; for example an efficient harvesting method could suppress the growth of contaminant microbes that would consume the hydrogen. Other issues involved implementation of large-scale systems and improving public perception. Photofermentative systems would generally generate CO_2 though breaking down organic molecules, which could cause concerns over the politics of CO_2/sustainability/greenhouse gases. The likely use of genetically modified organisms could also cause public concerns, though genetically modified microbes have not had the same public perception issues as genetically modified plants.

A number of barriers were discussed; those that received votes are shown in Table 3. The top barrier was the contamination of cultures by other organisms. Hydrogen is a universal electron donor and many microbes, not just methanogens, will scavenge it, and integrated systems and the use of certain feedstocks may increase the opportunities for contamination. Participants noted this barrier is common to all the biological production methods. Biorefinery integration, utilization of light/dark cycles and light utilization were identified as the next three most important challenges. Light utilization by the microbes is limited as their light saturation levels are well below full sunlight, and is largely affected by the light antenna. Alterations in the antenna may also change the stoichiometry of the photosystems and distribution of photocells. PNS bacteria are metabolically versatile but there is limited understanding of the metabolism changes during the light/dark cycle. This lack of information is a challenge to developing optimal reactor designs, as in addition to the metabolic aspects, the light/dark cycle can affect issues that could impact reactor design such contamination and stability, as the microbes can use the dark cycle to repair photosystems. Integration with biorefineries, which convert biomass feedstocks into one or more products, would provide a source of organic compounds for the feedstocks, for example from biorefinery wastewaters, the on-site production of organic molecules such as glycerol or acetate, or the biomass from other bioreactors. One participant brought up an integrated system model from the 2012 FCTO Multi-Year Research, Development and Demonstration Plan (MYRD&D; found at http://www1.eere.energy.gov/hydrogenandfuelcells/mypp/pdfs/production.pdf) with three systems: a photofermentative system utilizing infrared light, a photolytic system utilizing visible light, and a fermentation system that breaks down the biomass from the other two systems, producing small organic molecules that can be fed back to the photobiological systems. The participant noted that this works well on paper but is difficult in practice given the multiple processes involved.

Light availability and reactor design was the next barrier – the reactor affects the light available to the culture by the design and what the reactor is made of, such as optically-transparent materials. Reactor design will also be important in integrating systems that may supply feedstocks or remove waste products and make the system sustainable. The last three barriers that received votes all involve metabolism and the pathways to hydrogen production, related to the conversion of electrons from one form to another, the nitrogenase competition of N_2 and H^+ (which preferentially results in NH_3 production rather than H_2 production), and understanding how hydrogen metabolism integrates with aerobic metabolism.

Table 3. Photofermentative Hydrogen Production Barriers*
• Preventing contamination – i.e. H_2 consumption by other organisms (7) • Biorefinery integration (5) • How to utilize light/dark cycles (5) • Light utilization (5) • Light availability/reactor design (4) • Conversion of electrons from one form to another (3) • Nitrogenase competition of N_2 + H^+ (2) • Understanding how H_2 metabolism integrates with aerobic metabolism (1)

* Each participant voted for up to five different barriers. Total votes are indicated by the blue numbers. Only topics that received at least one vote are shown; in some cases the barrier topics were worded as solutions by the breakout participants.

The participants identified a number of R&D topics and those that received votes are listed in Table 4. When separating the topics into the different time periods the participants determined that none of the identified R&D fit into the “Long-term” category and instead made a near/mid-term category. In the near-term, R&D to make photosystems more efficient received the most votes, followed closely by R&D to gain a fundamental understanding of light/dark cycles. The last near-term R&D need to receive votes was to define applied metrics, do Life Cycle Analysis (LCA)/TEA studies using experimental data to the extent possible to test if the developed strains

could be economically viable and move from fundamental research to applications. In the near/mid-term, metabolic modeling received the most votes, followed by bioprospecting and genetic tools. Metabolic modeling would address many of the barriers relating to metabolic and energy pathways. Bioprospecting, or searching the environment for strains with desired traits, could identify species that have improved activities, though a challenge may be that some of the desired traits may not be evolutionarily beneficial. Genetic tools are addressed in more detail in the final day's discussion.

The top mid-term R&D was related to developing complete systems, including the top voted R&D overall, integration of the photofermentation systems with biorefineries, followed by reactor design and moving the technology to an applied scale (currently no system larger than 1000 L has been tested). It was suggested that methods to prevent contamination could involve reactor engineering as well as microbiology; for example, using thermophiles and keeping the reactor at high temperatures would help suppress most contaminant species. The last two R&D topics were to increase the rate of hydrogen (mainly through increased flow of electrons to the nitrogenase) and investigation of the regulatory networks of nitrogen metabolism.

Table 4. Photofermentative Hydrogen Production R&D*	
Near-term	• Making photosystems more efficient (5, 3) • Fundamental understanding of light/dark cycles (4, 2) • Define applied metrics, integration with experiments (1)
Near/Mid-term	• Metabolic modeling (4) • Bioprospecting and genetic tools (2)
Mid-term	• Ways to integrate biorefineries (6) • Reactor design (4) • Move technology to applied scale (3) • Methods to prevent contamination (2) • Increase rate of hydrogen production, i.e. electrons to nitrogenase (2) • Regulatory networks (1)

* Each participant could vote for up to five different R&D topics across all timeframes; the total of these votes are indicated by the green numbers. Each participant could vote for one top R&D topic in the near-term; the totals of this near-term topic vote are indicated in red. Only R&D topics that received at least one vote are shown in this table.

Biohybrid Systems and Enzyme Engineering for Hydrogen Production

Biohybrid systems were defined as *in vitro* systems that utilize at least some biological components for hydrogen production, not hybrid organisms, for the purposes of this workshop. General biohybrid system advantages include removing the microbial metabolic "overhead cost" requirements of keeping the cells alive and growing, having direct electron transfer (not diffusion dominated), the potential for improved solar-to-hydrogen (STH) or quantum efficiencies, higher turnover numbers, and increased energy efficiency without the loss of the proton motive force. Compared to photolytic systems, possible advantages include the potential to avoid hydrogen/oxygen separation requirements and oxygen inhibition. Compared to fermentation systems, biohybrid systems could be more flexible in terms of electron sources, mixing and matching sources depending on consumption.

Issues that need to be addressed were discussed next. System issues included extracting enzymes, scaling (specifically the cost of materials including the enzymes, which would depend on the enzymes needed), and better catalysts, especially for multi-subunit enzymes. Issues involving the integration of components included solvent incompatibility and stability of the coupling for water splitting, whether PSII or an inorganic system. Issues relating to the reaction were donor side turnover and the coupling of electron transport. Coupling of electron transport

issues are related to both the proton coupling from the reducing side, and the fast back-recombination, which is an unfavorable outcome that biological systems are able to control well.

Directed assembly and delivery of the enzymes, and self-repair/self-regeneration issues were discussed. Because systems are *in vitro* they may not be self-regenerating, but if the enzymes are inexpensive enough they could just be replaced. The lack of structure-based information to create a robust framework is an issue for enzyme engineering. Solar efficiency issues will depend on what will be used for light harvesting, and may be an attribute or deficiency. Lastly, learning from the best industry practices from photovoltaic technologies and transferring those advances to biohybrid systems will be important to advancing this technology.

A number of barriers were discussed and grouped into general categories. Table 5 lists the votes received by the various categories. The barrier with the most votes was lack of system integration and knowledge of design rules. The limited knowledge of options to integrate components in electron transfer is a barrier because there are many different strategies for this, and different ways to connect the pieces together.

The next two barriers were based on enzyme determents and material costs. Identification of structural determinants of catalytic efficiency and stability of enzymes is needed to better understand the basic mechanisms of the photochemical module electron transfer to proton-coupled electron transfer at the hydrogen metal catalyst site and to elucidate the fundamental design principles needed to make new or improved systems. The current lack of a robust knowledge base for enzyme engineering, and cost-effective biocatalyst production and regeneration systems are also related barriers.

Material costs, based on raw material costs and manufacturing and scalability is a barrier, with enzyme costs being the main costs for all systems. Bio-derived materials have high costs and there is currently a lack of cheap, robust and massively scalable cell-free systems to produce the enzymes or a way to make synthetic enzymes. Enzyme and photosystem cofactor costs are another part of the material cost barrier, given the lack of a scalable, low-cost production method. The limited half-life of bio-derived materials also affects costs; more studies into sustainable self-regeneration with reasonable cost, immortal enzymes/catalysts, and novel materials that mimic cell membranes for enzyme stability/activity are needed. Development of soft-matter, self-healing supramolecular scaffolds for hybrids and scaffolds to stabilize molecular entities would enable integration and systematic understanding.

The lack of TEA/LCA comparison for biohybrid systems tied with the lack of *in situ* diagnostics at the molecular scale for biohybrids as the next highest voted barrier. *In situ* diagnostics are needed to be able to track where components of biohybrid systems go and why, for example assessing local electron utilization to determine if cofactors are being lost. Diagnostics would enable better integration of the systems. Though biohybrid systems would seem to have advantages for *in situ* analysis, the technologies are not yet developed and better computational design tools for hybrid systems are needed. The last barrier is conversion efficiency, which is in part limited by ineffective coupling between components and is not limited to light conversion efficiency. Better integration of the components is needed to limit back reactions and improve long-term functions.

Table 4. Biohybrid Systems and Enzyme Engineering for Hydrogen Production Barriers*
• System integration and design rules (8) • Enzymes – identification of structural determinants of catalytic efficiency and stability (7) • Material cost – raw material cost, manufacturing and scalability (7) • Lack of TEA/LCA comparison for bio-hybrid systems (5) • Lack of *in situ* diagnostics at the molecular scale for bio-hybrids (5) • Conversion efficiency (4)

* Each participant voted for up to five different barriers. Total votes are indicated by the blue numbers. Only topics that received at least one vote are shown; in some cases the barrier topics were worded as solutions by the breakout participants.

The R&D topics that received votes are listed below in Table 6 In the near-term, the top R&D topic was the study of enzyme stability and activity and using bioprospecting as a foundation for exploring the environmental diversity of enzyme primary structures and natural diversity; it was noted that the construct framework needs to be identified. Along with TEA was the need to establish performance metrics. It was noted that exploring synthetic/biosynthetic processes for enzymes would also give an opportunity to expand functionality. Other near-term R&D involved systems integration and analysis: basic electron transfer studies for integration, TEA to assess cost sensitivities, test bed development, and exploring synthetic/biosynthetic processes for enzymes production, as well as more fundamental studies and enzyme surface design for inorganic coupling.

In the mid-term, the top R&D topic was design rules for enzyme engineering, based on structure activity relationships (SAR). For reactor design, it was noted that one issue is how to bring engineering into the system designs for conversion on a large scale, and that systems engineering need to be addressed before scale-up and manufacturing. As part of the enzyme expression and cost reduction R&D, the enzyme production will need to reach a large-scale. Other discussion suggested that a fully integrated system at pilot/prototype scale would need to be developed in the mid-term. In the long-term, R&D included improving biological components for coupling and efficiency through evolution, scalable, low-cost enzyme production, and commercial deployment. While the photolytic water splitting system topic did not receive votes, it was noted that this is a big goal.

Table 5. Biohybrid Systems and Enzyme Engineering for Hydrogen Production R&D*	
Near-term	• Enzyme stabilization and activity studies and bioprospecting - foundation for understanding environmental diversity of enzyme primary structure and natural diversity (8, 6) • Basic electron transfer studies for integration (4, 2) • Technoeconomic analysis to assess cost sensitivities (5) • Test bed development (2) • Fundamental studies (1) • Enzyme surface design for surface (inorganic) coupling (1) • Exploring synthetic/biosynthetic processes for enzymes (1)
Mid-term	• Design rules for enzyme engineering (SAR-SAR) (5) • “Reactor” design (2) • Test bed integration (2) • Systems integration (1) • Enzyme expression/cost reduction (large scale) (1) • Rules for increasing conversion efficiency (1)
Long-term	• Evolving improved biological components for coupling and efficiency (2) • Enzyme production from low-cost process, scaling (1) • Commercial deployment (1)

***** Each participant could vote for up to five different R&D topics across all timeframes; the total of these votes are indicated by the green numbers. Each participant could vote for one top R&D topic in the near-term; the totals of this near-term topic vote are indicated in red. Only R&D topics that received at least one vote are shown in this table.

Photobiological Hydrogen Production Final Discussion

Photobiological Hydrogen Production Conclusions

Bioprospecting to take advantage of natural diversity
- Hypothesis- and target-driven studies could allow researchers to identify and understand potentially useful enzyme variations generated by nature that may allow for improved hydrogen production

Improved understanding of electron flux
- Understanding the energy flows within cells, both those that directly lead to hydrogen production, and those that can indirectly affect production, would allow better targeting of genetic engineering

Tool development
- Tools for manipulating many hydrogen-producing strains are currently limited, and developing these would enable researchers to improve strains of interest

Potential methods to avoid oxygen co-production
- Co-production of oxygen during photolysis damages the hydrogen-producing enzymes and causes practical problems for safety and hydrogen harvesting, so developing methods that avoid simultaneous co-production would be beneficial

A full group discussion followed the report-out from each breakout group. Because bioprospecting was mentioned in all three report-outs, the discussion opened with that topic. Bioprospecting generally looks at large number of environmental strains, and can be based on strains that have been isolated and whose growth and metabolism can be tested directly or more recently on metagenomic data. The advantage of this is that in nature, microbes have had billions of years and many different conditions to generate variations of hydrogenases and nitrogenases, which have been "tested" through natural selection, with far more variations than researchers have or even could study in depth in the lab. Bioprospecting could identify strains with significantly improved hydrogen production characteristics, or through comparison could identify common traits that may be beneficial in certain conditions. For example, sequence variations commonly found in hot springs organisms could be involved in temperature tolerance. The more diverse the sample set is, the more robust the search could be. For this to be effective for applied research, the searches would need to be hypothesis driven, with targeted searches with effective screening or selection methods. In screening methods, all samples are tested for the presence, absence or degree of a characteristic, while selection methods subject all samples to tests which result in only those samples with the characteristic of interest being selected and identified; therefore selection methods can often be used to process larger samples. Another issue is identifying appropriate sources for the samples. It was noted that there are many existing metagenomic libraries, and for those the field "prospecting" aspect have been completed, with the remaining work being to develop hypothesis-driven analysis of the data, including *in silico* sequence analysis; for example, the DOE Office of Science has had gene discovery efforts that might be leveraged. Locations that might have naturally selected for high hydrogen production include underground microbial communities where hydrogen metabolism is already occurring.

Another topic that was discussed in multiple breakout rooms was the importance of electron flux. The flow of electrons generated from water splitting or other processes to the hydrogenase or nitrogenase plays an important role in the rate of hydrogen production. Because other cellular pathways and reactions can use the same electron carriers once they are generated, maximizing the flow to hydrogen production will require ways to direct the electrons to the desired enzymes, reduce or eliminate the competing pathways, or both. A related issue is the ultimate products of the metabolic processes – currently once organic molecules are broken down to 2- or 3-carbon molecules, they are generally not broken down further and known metabolic pathways will not generate more hydrogen, but to maximize hydrogen production the goal would be to break down all carbohydrates to CO_2 and H_2. Some ways to do this would be to stop pathways to the 2- and 3-carbon molecules such as acetate,

reengineer glycolysis, or use a hybrid system where the acetate goes to another organism, such as a strain of PNS bacteria, which can use it to produce hydrogen.

Ultimately the goal would be to have all the desired characteristics in one enzyme, though in many cases there would be trade-offs between traits, which might be determined from knowledge-based designs or need to be experimentally tested. Currently desired qualities include oxygen tolerance, thermal tolerance, stability, ability to interact with the cell's systems, and high or fast activity. It is possible that all these traits could be found in a currently undiscovered organism, but if not then they could be engineered into a single organism, along with any required maturation genes needed to properly assemble the hydrogen-producing enzymes.

The problems of hydrogen/oxygen mixtures was also discussed – in addition to the oxygen sensitivity of hydrogen-production enzymes, other concerns include the safety issues and the need to develop systems for gas separation. Potential methods to avoid co-production were discussed. The sulfur deprivation method results in no net-production of oxygen by reducing the rate of oxygen evolution from photosynthesis to a level that is immediately consumed by respiration, but at the cost of reduced photosynthetic activity. One method would be to absorb oxygen, for example with myoglobin, which absorbs oxygen at low partial pressures. One consideration would be how the oxygen would be unloaded so that the material could continuously absorb oxygen. Another option would be to find a pathway or enzyme that would consume the oxygen. Alternative oxidases, which will allow for high rates of PSII activity, might work for this. Because high PSII activity levels would produce high levels of ATP, it might be more appropriate for nitrogenase-based systems, as ATP is needed for the nitrogenase reaction. It was noted that *C. reinhardtii* does have an oxidase, but it uses electrons that could otherwise be used for hydrogen production.

Several aspects related to the location and activity of the hydrogen production enzymes were discussed. The proportion of the enzymes that are active in a cell is not well understood, and some experiments have shown that simply increasing the amount of protein does not increase activity. Similarly, the location of active hydrogenases in cells is not fully understood, though there is evidence that the organization may be different under varying conditions, such as nutrient replete compared to sulfur deprived cells. Ways to potentially increase production rates include compartmentalizing the enzymes, or to design and engineer buffer domains to protect the enzymes from damage. The breakdown process for hydrogenases has been found to be complex *in vivo* and identification of the proteins involved in that breakdown could provide a way to control the turnover or breakdown of the enzymes.

Non-Light Driven Biological Hydrogen Production

Non-Light Driven Biological Hydrogen Production Presentations

Dr. Bruce Rittmann (Arizona State University), "*Non-Photosynthetic Biohydrogen – Overview of Options*."
Dr. Rittmann, the panel leader, opened the session with an overview of non-light driven biological hydrogen production methods. Dr. Rittmann outlined the three non-light driven pathways to produce renewable hydrogen from biomass: fermentation; MEC; and methanogenesis followed by reforming. He noted that these pathways, although classified as "non-light driven", are really indirect photosynthetic biohydrogen since the biomass ultimately came from photosynthesis.

In his overview of fermentation, Dr. Rittmann recognized it as a very mature and well-studied technology, especially with respect to sugars, with the advantages including simple bioreactor designs and the ability to use more complex biomass feedstock as input to the fermentation process. As for the disadvantages, low hydrogen yield is among the most concerning. Hydrogen is produced only from the oxidation of the reduced ferredoxin and formate with the electron equivalents going to acetate, ethanol butyrate, lactate or propionate being lost for potential biohydrogen. As a result, Dr. Rittmann stated that 2 mol H_2/mol glucose is accepted as the practical maximum hydrogen yield from fermentation and actual hydrogen conversions are often even less. Other disadvantages mentioned were the impurity of the evolved hydrogen (from other fermentation products such as CO_2) and the high biochemical oxygen demand (BOD) of the liquid effluent as well as the need for a follow on step to allow for increased hydrogen production from the waste stream.

The second part of Dr. Rittmann's presentation focused on MECs which are essentially modified microbial fuel cells (MFC) that produce hydrogen instead of electrical power. In MECs, the anode side is the same as an MFC with the main difference being on the cathode side where, for an MEC, oxygen is excluded so the electrons reduce the protons and make hydrogen with the help of a small applied voltage. Dr. Rittmann stressed that the MEC makes hydrogen production a respiratory process not a fermentation process, making it a simple process with the potential for nearly 100% hydrogen yield. In addition to this advantage, the hydrogen produced is nearly pure after the water vapor is removed and the liquid effluent can have a low BOD. In terms of the disadvantages related to MECs, Dr. Rittmann stated that this is still an emerging technology that requires an applied potential, which needs to be minimized to bring down energy costs. Further, the costs related to the electrodes, membranes and controls will need to be addressed. Finally, the anode respiring bacteria (ARB) which act as the catalyst for substrate oxidation, use simple substrates like organic acids as feedstocks, instead of complex molecules like those found in unrefined biomass. While not being able to use some biomass feedstocks is a drawback, it makes MECs a nice follow-on to fermentation processes, for increased net hydrogen yield.

Dr. Rittmann finished his presentation by very briefly discussing methanogensis followed by methane reforming as a pathway for hydrogen production. He stated that it is a mature technology and has a similar conversion efficiency as compared to MEC.

Dr. Charles Dismukes (Rutgers University) "*Autofermentative Biological Hydrogen Production by Cyanobacteria*."
This presentation was originally scheduled for day two but was moved to the end of day one. Dr. Dismukes started his presentation by introducing what he referred to as the "new player" on the cyanobacterial scene, a formate

hydrogen lyase hydrogenase that was not thought to be in the strain until recently when it was found in the genome. This is an NAD-dependent hydrogen production pathway that gives the opportunity to couple formate and hydrogen production. Dr. Dismukes also discussed a recently described oxygen insensitive uptake enzyme in the *Ralstonia* bacteria that could potentially offer some learnings that could be applied to the cyanobacteria system.

For cyanobacteria, Dr. Dismukes stated that glycogen is the preferred carbon source for fermentative hydrogen, and glycolysis is the preferred catabolism pathway under fermentative conditions. However, the glycolysis pathway for catabolizing the glycogen is wasteful when it comes to maximizing the reductant. Therefore if the glycogen catabolism can be rerouted through the alternative oxidative pentose-phosphate (OPP) pathway, hydrogen production could be maximized.

Dr. Dismukes then went on to discuss the work being done in his lab which has focused on stimulating hydrogen production by "milking" the system. By capturing the hydrogen as it is produced, the reduction/oxidative balance shifts, shifting the pathway to OPP. As a result, lactate, formate, and ethanol production are decreased, resulting in ~40% increase in hydrogen production. Dr. Dimuskes ended his talk by summarizing the status of autofermentative hydrogen production in cyanobacteria reiterating some of the limitations and possible solutions to them.

Dr. Jason Ren (University of Colorado Boulder) *"Microbial Electrolysis Cells (MECs) for High Yield Hydrogen Production from Biodegradable Materials*." Dr. Ren gave an overview on MECs covering the features and current status, challenges and opportunities, and what he believes to be the greatest R&D needs for MECs as a platform for hydrogen production. He began by discussing the different types of microbial fuel cell-based technologies (MxCs) with a focus on how an MEC operates and its potential to reach >90% recovery from a variety of organic substrates such as cellulosic biomass, fermentation products, and wastewater. He explained that one of the benefits to this technology is that it is not dependent on hydrogenase, ferredoxin, or NADPH. Dr. Ren also highlighted that the progress in this field with respect to hydrogen yield and production rate over the last eight years since the technology was discovered are quite impressive. Other features noted by Dr. Ren include the potential for high purity hydrogen directly from the MEC and the advantages of integrating MECs with a fermentation process to help improve overall hydrogen yield.

With respect to the challenges faced by MEC technology for hydrogen production, Dr. Ren discussed the need for an external power source. In practice, an applied potential of more than 1 volt is needed to drive the reaction. However, he also noted that renewable sources such as MFCs, salinity gradients and waste heat have been used to provide additional energy for H_2 production in MECs. Hydrogen sinks such as hydrogen consumption by methanogenesis are also challenges that need to be overcome in order for MECs to be a viable technology for low-cost hydrogen production. Dr. Ren closed his talk with what he believes to be the greatest R&D needs in this area: development of new materials and reactor configurations to increase hydrogen production rates and reduce system and operational costs; exploration of microbial and engineering approaches to reduce or remove competitive hydrogen consumption and increase hydrogen harvesting rate or utilization; and efforts geared toward system scale-up and integration with other complementary processes.

Dr. Adam Guss (Oak Ridge National Laboratory) "*Metabolic Pathways and Metabolic Engineering*." Dr. Guss opened his talk by stating that metabolic engineering is a way for researchers to modify organisms to get the organisms to do what they want them to do. One successful example of this is the metabolic engineering of *Clostridium thermocellum* for cellulosic ethanol production, through which gene deletion and heterologous gene expression have led to an increase of ethanol production of more than twice that of the unmodified species. Dr. Guss stated that such successes require a foundational understanding of the metabolic pathways.

Dr. Guss discussed two strategies that can be used in metabolic engineering. The first is to start with an easily modifiable industrial organism that can be engineered for the desired function, which requires a deep understanding of the pathways to be heterologously expressed and the ability to control the expression and mitigate the toxicity of the intermediates and products to the host system. The second option would be to start with an organism that already has the unique, desired capabilities. The genetic systems would then need to be developed, and an understanding of the metabolism, gene regulation, etc. acquired in order to engineer the species to make only the compound of interest (i.e. hydrogen) and make the organism more robust.

As for the status of the technology, Dr. Guss stated that developing new genetic systems is difficult, yet feasible. DNA synthesis is inexpensive and the cost continues to come down, shifting the emphasis from tool development to “ideas”. Metabolic models and computational tools are becoming more advanced and could be quite informative to future strategies. The incomplete knowledge base and understanding of enzymatic pathways is a barrier that will need to be addressed as will the complexity of the systems and the limited capability of the current models. Dr. Guss explained that key needs in the near-term are success stories to keep the field moving and for research to address applied metrics to show the value of the effort. In the mid-term, he stressed the importance of exploring the basics of the native metabolic and regulatory pathways to increase the foundational understanding and for completely new approaches to be explored as the best approach may yet to be defined.

Dr. Melanie Mormile (Missouri University of Science & Technology) “*Bacterial Fermentative Hydrogen Production*.” Dr. Mormile concluded the panelist presentations for the non-light driven biological hydrogen production session with a talk on bacterial fermentation focused on pre-treatment options and their implications. She discussed three possible lignocellulosic pre-treatment methods used to separate and degrade soluble lignin/hemicellulose from insoluble cellulose. Steam-blasting is a current option; however it is heavily dependent on fossil fuels as an energy input and releases phenols, carboxylic acids, and salts which inhibit fermentation. Acidic treatment is similar in that it is also fossil fuel dependent and releases phenols and phenol aldehydes as well. The last method discussed was alkaline treatment which results in the highest ethanol yield of the three. Phenols are still produced, but at lower concentrations so neutralization remains an issue if traditional microorganisms are to be used for the fermentation process. However, Dr. Mormile suggested the possibility of using haloalkaliphilic bacteria that naturally produce hydrogen as a potential way to eliminate the treatment step needed to clean up alkaline treated biomass prior to the fermentation step. The example she gave was *Halanaerobium hydrogeniformans*. With optimum growth conditions of pH 11 and 55°C, and a salt tolerance of 7.5%, it has already been shown to produce hydrogen from switch grass liquor and straw.

In conclusion, Dr. Mormile reported on the status of bacterial fermentation hydrogen production and noted that the theoretical yield of 4 moles H_2/mole of glucose has not been demonstrated to date and recognized the need to increase the substrate conversion, posing the question “Is it possible for a biological system to extract all 12 hydrogen atoms from glucose?”. The theoretical yield of 4 moles H_2/mole of glucose could possibly be surpassed through metabolic engineering of new metabolic pathways, or by using a hybrid system approach. Increasing the hydrogen yield through “milking” the system, using a microbial consortia and optimizing bioprocess parameters were also key research needs according to Dr. Mormile. Other barriers discussed were the lack of optimal bioreactor designs and the cost of the raw materials, including the cost of treating the waste and pretreatment costs.

Non-Light Driven Biological Hydrogen Production Panel Discussion

The focus of the panel discussion was on MECs and the potential to integrate these systems with fermentative processes. The first item discussed was the biofilm within the MEC used to transfer electrons and its stability. It

was stated that these films have been shown to work continuously for months. The biofilms are well understood, with the main two issues being (1) they can get clogged up, and (2) dealing with the proton build up resulting from e- production within the biofilm, which is a mass transport issue. The scale-up of MECs was also discussed and a market study done at University of Colorado showed that these can be used in centralized systems, like wastewater treatment plants, as well as semi-central and distributed systems, with the systems most likely being modular. Typically these systems, which have borrowed most of the materials and design from Proton Exchange Membrane fuel cells, use carbon and not platinum or Nafion® because of their cost. When asked whether the cathodic performance would benefit from a catalyst, the panelist agreed and said platinum is currently the best, but, again said cost is the issue. In terms of an MEC's ability to operate with real-world waste versus model substrates used in the lab, the panelists said this is not an issue, the issue is matching the MEC with the fermentation product and that with more complex feed streams, there are competing routes to be concerned about, especially those leading to methane.

When the discussion shifted to the integration of MECs with fermentation, a participant noted that for the highest net hydrogen production, the goal of fermentation shifts from producing hydrogen to producing acetate as a feedstock for the MEC and asked if shifting to acetate is feasible. Dr. Mormile stated that the main product from her lab's process is acetate, and clarified that the pretreatment uses sodium carbonate only, not enzymes, to pretreat the biomass. The ability to tune the residence time (typically between 2-8 hours) and the surface area within the MEC was also discussed with regards to integrating the systems.

Non-Light Driven Biological Hydrogen Production Breakout Discussions

Fermentative Hydrogen Production

The workshop participants involved in the fermentative hydrogen production breakout group began their session discussing some of the major issues associated with the ability of fermentative systems to achieve low-cost hydrogen production. The issues discussed included biomass pre-treatment and cellulosic degradation, as well as the need for a bioreactor design to optimize hydrogen production. The public's perception of hydrogen as being dangerous was also mentioned, however, the group felt this was out of scope for this workshop.

Developing a Consolidated BioProcessing (CBP) platform, which employs cellulose degrading microbes that produce their own cellulase cocktail to hydrolyze the cellulose, and simultaneously ferment the products to hydrogen is a major challenge. Robust microbial consortia were said to be key to addressing the pretreatment issue as well as removing the lignin from the biomass. Overall it was recognized that the pretreatment process needed to be cheaper, better and faster than the current technology.

The discussion quickly moved from issues to barriers. The results are summarized in Table 7. Barriers related to the need for a better understanding and further development of genetic systems and a reactor design optimized for hydrogen production, including embrittlement mitigation, and sizing and scale up of the reactor had the most votes with 8 each. The lack of systematic knowledge on fermentative hydrogen production and a need for a better understanding of the ability to convert biomass received several votes as well. To address this barrier, bio-prospecting to find better microbes to ferment both the cellulose and hemicellulose is needed. The low molar yield, the lack of available and affordable feedstocks (including high transportation costs), and the processing of the non-cellulosic substrates did not receive as many votes, but were identified as barriers that need to be addressed.

Table 6. Fermentative Hydrogen Production Barriers*
• Understanding and development of genetic systems. (8) • Reactor design. (8) • No knowledge base – lack of systematic knowledge on fermentative H_2 production. (7) • Better understanding of biomass convertibility. (6) • Low molar yield. (4) • Lack of available and affordable feedstocks. (4) • Processing of non-cellulosic substrates. (3)

* Each participant voted for up to five different barriers. Total votes are indicated by the blue numbers. Only topics that received at least one vote are shown; in some cases the barrier topics were worded as solutions by the breakout participants.

The break-out group then identified the R&D they felt are needed to overcome the major barriers, separated them in-terms of near, near/mid, mid, and long-term needs. The results are summarized in Table 8. The group nearly unanimously agreed that in the near to mid-term developing genetic tools and microbial manipulation are the most critical R&D to enable low-cost hydrogen from fermentation, both to improve production and to improve tolerance of the system conditions. In the near-term the group identified R&D leading to a better understanding of the hydrogenase as the greatest need. It is always valuable to better understand the catalytic properties, subunit compositions, and structure of the enzyme so it can be further improved through the use of genetic tools. Some examples would be better understanding how O_2 inhibits the hydrogenase of interest so that strategies could be developed to cope with or mitigate the inhibition. Building better consortia of microbes able to convert compositionally diverse feedstocks to hydrogen was also identified as a near-term need. It may be more feasible to have a group of microbes working together than engineering a single microbe to degrade all types of substrates within a feedstock since some microbes cannot be readily engineered, and often it might compromise other pathways. Tailoring biomass development specifically for hydrogen production received several votes as well and was identified by one participant as the most critical R&D need. In the long-term, systems integration and demonstration was found to be the greatest need. Other R&D identified included bioreactor design and material development at the lab, pilot and eventually small industrial scale, and TEA and LCA for various feedstock and system integration options.

Table 7. Fermentative Hydrogen Production R&D*	
Near-term	• Better understanding of hydrogenase (4) • Bioreactor design, Lab (10L) to Pilot scale (100L) (3) • Bioreactors – Microbial consortia and integration (3) • Bioreactor material development (1)
Near/Mid-term	• Tailor development of biomass for H2 production (4,1) • Genetic tools/Microbial manipulation (9,6) • TEA/LCA for various production options/system integration options (2)
Mid-term	• Bioreactor design, Pilot (100L) to Small Scale Industrial (1500L) (1) • Systems Integration – Systems biology and fermentable microbes (2) • Synthetic biology (2)
Long-term	• Systems Integration, Demonstration (4)

* Each participant could vote for up to five different R&D topics across all timeframes; the total of these votes are indicated by the green numbers. Each participant could vote for one top R&D topic in the near-term; the totals of this near-term topic vote are indicated in red. Only R&D topics that received at least one vote are shown in this table.

Hydrogen Production by MxCs

Several issues were identified by the MxCs break-out group, the first being the need for adequate proton carriers to transfer protons from the anode over to the cathode. What is needed is a cheaper, lighter alternative. Proton conducting polymers were mentioned as a possibility, but they are not sufficiently electronically conductive. Related to the proton transport issue is pH. At a neutral pH, which is an optimum condition for many microbes, there are few protons present for facile proton transport. To have optimal growth and activity at the anode, acidophilic and thermophilic organisms are needed that could work at the low pH and higher temperatures of the anode environment. Inexpensive anodes, increasing the membrane surface area for scale-up, and a design that would bring the electrodes closer together were also identified as issues for hydrogen production by MxCs. Issues with the cathode were discussed and included lowering the overpotential and developing better catalysts for improved H+ reduction. A major issue identified was methanogens, which are a greater problem on the anode side and can drastically decrease hydrogen yields in MxCs. Feedstream quality was also discussed as a factor influencing MxC performance as it could help control biofilm growth/thickness, and a defined substrate or substrate mixture would be easier to study and develop a system to process (hence the attractiveness of combining MxCs with a fermentation process). The buffer capacity of the feedstock is also important for these systems. A feedstock containing a protein is desired as it releases ammonia which takes up the protons and helps alleviate the proton build-up/mass transport issue associated with MxCs.

After the initial discussion of the issues associated with hydrogen production from MxCs, the participants then identified, discussed, and voted on the major barriers to low cost hydrogen production. The summary of the barriers and voting results are laid out in Table 9. The group identified six major barrier areas and then further examined sub-topics of these barriers. The area receiving the most votes was microorganisms, specifically identifying microorganisms able to function at lower pH. The high cathode overpotential was another barrier identified, specifically the need to develop catalysts/electrodes that can reduce protons to hydrogen at a lower cathode potential. This, in turn, would improve system efficiency. Proton transport, in particular increasing the rate of transport, and new system configuration, in particular system scale up, were identified as important barriers for this technology. System integration with other processes such as fermentation and identifying the source for potential loss are also barriers identified.

Table 8. MxC Hydrogen Production Barriers*	

• Testing of potential microorganisms
  ○ Microorganisms – function at lower pH needed (6)
  ○ Microorganisms – function at higher temp needed (2)
  ○ Controlling bad side reactions (1)
• Cathode overpotential
  ○ Catalyst/electrode to reduce cathode potential loss (4)
  ○ Low-cost cathode materials/structures (2)
  ○ Better cathode catalysts (1)
	• Proton transport
	  ○ Slow H+ transport (4)
	  ○ Low-cost ion exchange membrane needed (2)
	  ○ Proton transport to cathode (1)
	• New system configuration (1)
	  ○ Scale up (4)
	  ○ Low anode surface area (2)
	  ○ Control systems – concentration, flow (1)
	• System integration with other processes
	  ○ Fermentation targeted to MEC feed (2)
	• Identify source of potential loss (2)

* Each participant voted for up to five different barriers. Total votes are indicated by the blue numbers. Only topics that received at least one vote are shown; in some cases the barrier topics were worded as solutions by the breakout participants.

The panel was then tasked with identifying the most critical R&D needed to overcome the barriers identified. For the near and mid-term needs, R&D receiving at least one vote are summarized in Table 10. Only three long-term R&D needs were identified and did not receive any of the votes but are included in the table as well. R&D associated with improving the cathode and reducing its overpotential received the greatest number of votes in terms of important R&D in the near-term. In line with improving the cathode to reduce the overpotential, the need to precisely characterize performance loss, identifying the mechanisms related to potential losses and quantifying each step of potential loss, also received a large number of votes. Efforts to develop scaled-up designs to look at how stacking the cells will affect efficiency and long-term cell stability, and determining ideal designs and configurations that would enable high-volume hydrogen production were identified as near-term R&D needs as well. Also required in the near-term are standardized metrics in order to compare systems and designs and determine their likelihood to meet hydrogen production goals. Integration with fermentation systems to optimize performance will also be necessary in the near-term. As with many of the breakout sessions, TEA was identified as a near-term R&D need as well.

The need for improved anode organisms that can perform in a low pH (~5) and high temperature (~60°C) environment was found by the panelists to be the number one focus area for mid-term R&D. R&D for improved proton transfer, improved cathode catalysts, and scale-up were also identified as R&D needed in the mid-term. In the long-term, the panelists felt R&D should be focused on material and system durability and stability, improving anodes through microbe engineering, and developing a better understanding of relevant microbial metabolisms.

Table 9. MxC Hydrogen Production R&D*	

Near-term	• Cathode improvements (4, 4)
	  ○ Cathode to reduce overpotential, higher-surface-area cathode (4 green votes)
	• Precisely characterize performance (5, 1)
	  ○ Precisely quantify each step of potential loss that leads to overpotential (2, 1)
	  ○ Characterize mechanisms of "potential" loss (sources of overpotential) (3)
	• Fermentation + MEC integration to optimize performance (4, 1)
	• Standardized metrics (4, 1)
	• Develop scale-up designs (6)
	  ○ Study effects of "stacking" on efficiency and long-term stability of cells (4)
	  ○ Investigate reactor system configurations for large module sizes and high vol. productivity (2)
	• Improved proton exchange membranes (2)
	  ○ Low-cost cation and anion exchange membrane (1)
	  ○ Better ion-exchange membranes (1)
	• Technoeconomic analysis (2)

Mid-term	• Improved anode organisms (3)
	  ○ Development of high-temp (~60°C) low-pH (~5) biofilm (2 green votes)
	  ○ New organisms for anode-acidophiles thermophiles (1 green vote)
	• Develop improved H+ transfer systems (2)
	• Develop improved cathode catalyst (2)
	• Scale-up – longevity, cost reduction (1)

Long-term	• Durability and stability • Improve anode with microbe engineering (metabolic/system synthetic) • Microbial metabolism understanding

* Each participant could vote for up to five different R&D topics across all timeframes; the total of these votes are indicated by the green numbers. Each participant could vote for one top R&D topic in the near-term; the totals of this near-term topic vote are indicated in red. Only R&D topics that received at least one vote are shown in this table.

Genetic and Metabolic Engineering for Hydrogen Production

The Genetic and Metabolic Engineering for Hydrogen Production breakout group began their session by identifying the major issues in this topic area. Improvement in the hydrogen to Biomass/Feedstock ratio was the first issue identified. Hydrogen molar yield needs to be improved for biological hydrogen production to be a viable pathway for low cost hydrogen. In order to do this, the ability to direct cellular metabolic activity toward hydrogen rather than other products must be developed. One participant said that nutrient costs account for at least 40% of the overall cost of hydrogen production. Engineering organisms to utilize lower-cost nutrients will be key to enabling low-cost hydrogen from biological pathways. Another issue identified is the need for strategies and tool development for large scale genetic manipulation specifically related to hydrogen production. Models that guide design, i.e. predictive modeling, for system pathways would be a useful tool to improve the efficiency and effectiveness of genetic and metabolic system engineering. Engineering system to allow for hydrogen production in the stationary phase will also be necessary for low-cost production of hydrogen.

Pathway competition that limits hydrogen production was found to be the number one barrier because it directly impacts the already limited hydrogen yield of biological systems. The inability to decouple growth from hydrogen production was also found to be a significant barrier for low cost hydrogen production because it leads to more complicated processes and reactor designs. The lack of rapid and systematic genetic tools was another major barrier identified by the group as these are needed in order to manipulate and optimize the systems for hydrogen production. A lack of sufficient predictive modeling and a lack of high throughput screening approaches for genetic testing which could help guide the experimental work is also a barrier that needs to be overcome as was mentioned in the “issues” section of the panels discussion. Other major barriers identified can be found in Table 11.

Table 10. Genetic and Metabolic Pathway Engineering for Hydrogen Production - Barriers*
• Pathway competition limits H_2-production (7) • Lack of rapid an systematic genetic tools / chassis development (6) • Inability to decouple growth from H_2-production (6) • Lack of sufficient predictive modeling (5) • Lack of high throughput screening approaches (3) • Low efficiency of spent biomass recycling (3) • High water demand in large volume H_2-production (3)

* Each participant voted for up to five different barriers. Only topics that received at least one vote are shown; in some cases the barrier topics were worded as solutions by the breakout participants.

Genetic tools to enable engineering for increased hydrogen production was identified as the most critical near-term R&D need in this session. The development of these tools does not directly increase hydrogen production, but is the necessary foundation needed to manipulate organisms for improved hydrogen production. Because low hydrogen yield is a major issue for low cost biological hydrogen production, the next most critical near-term R&D need was to improve yield through manipulating or knocking out pathways, including heterologous expression (expressing a non-native gene or part of a gene in the host organism) to optimize the organism for hydrogen production. Along with manipulating pathways, identification of co-culture consortia was also identified as an activity that could lead to increased hydrogen yield in the near-term. Stationary phase hydrogen production was also recognized as an area for near-term R&D efforts in order to increase hydrogen production in these systems.

In the mid-term, engineering metabolic robustness and developing combinatorial tools for genetically challenging organisms or co-culture consortia were identified as necessary R&D. Computational integration of biology and

engineering was identified as a long-term need in this area in order to more effectively and efficiently design, implement, and validate genetically and metabolically engineered systems for hydrogen production.

Table 11. Genetic and Metabolic Pathway Engineering for Hydrogen Production - R&D*	
Near-term	• Development of genetic tools / chassis to increase yield of H_2-production (5, 4) • Manipulate pathways to improve yield, including heterologous expression of pathways (3, 3) • Develop stationary phase H_2-production approaches (6) • Identification of co-culture consortia that will improve yield (4) • Development of high throughput screens (3) • Use metabolic flux analysis to track energy flow (2)
Mid-term	• Engineering metabolic robustness (5) • Combinatorial tools for genetically-challenging organisms or co-culture consortia (3)
Long-term	• Biology and engineering computational integration (3)

* Each participant could vote for up to five different R&D topics across all timeframes; the total of these votes are indicated by the green numbers. Each participant could vote for one top R&D topic in the near-term; the totals of this near-term topic vote are indicated in red. Only R&D topics that received at least one vote are shown in this table.

Non-Light Driven Biological Hydrogen Production Final Discussion

After the Workshop participants reconvened and the breakout groups reported on their findings, there was a full group discussion to discuss topics that may have been overlooked as well as common themes from the breakout sessions.

It was noted that hydrogen yield more than the production rate. Because the rate depends on many things such as the rate constant, the reactant supply, and is more than just the rate constant of a couple of enzymes, the participants felt at this stage yield is a more tangible/comparable metric. Further, the rate is actually often lower in a modified organism than in the original species, even though the modified species is likely the more optimal in terms of practical characteristics (e.g., improved tolerance to the system environment) and potential for genetic and molecular biology improvements (e.g., the ability to remove competing pathways).

The question was posed by the audience, “What is theoretical yield of hydrogen from 1 mole of glucose?” and the audience gave several answers to this question. The yield is 4 H_2 + 2 acetates using known fermentative pathways. When looking at the content of hydrogen in glucose, there are a total of 12 hydrogen atoms, so 6 H_2 moles of hydrogen theoretically. If one considers glucose + H_2O, the yield would be 12 H_2 + 6CO_2. *In vitro* systems have shown 11.4 moles H_2/(glucose +H_2O).

Non-Light Driven Biological Hydrogen Production Conclusions

Reactor design and scale-up

- Reactors will need to be designed to provide conditions that maximize microbial production, can be integrated into feedstock sources such as biorefineries, and support high production rates at large scale

Improved understanding of metabolism and energy flows

- Better understanding of metabolic pathways involved in feedstock breakdown and hydrogen production, and ion transfers in MxCs, particularly under relevant conditions, will be needed to guide optimization of the microbial characteristics and reactor design and conditions

Tool development

- Tools for manipulating many hydrogen-producing strains are currently limited, and developing these would enable researchers to improve strains of interest

The discussion then changed topics to fermentation and reactor design. As fermentation is an established technology, it was surprising to some that bioreactor design was one of the major barriers identified by the break out group. One challenge is using lignocellulose, a solid substrate, and having to stir it. There would be a huge benefit if the biohydrogen system could be run as a, or approaching a stationary phase (i.e. no net growth). During the growth phase of fermentation, large quantities of microorganisms are produced which both requires energy and metabolic resources that could be used for hydrogen production and also generates unwanted by-products along with the hydrogen production. If hydrogen production could be realized in the stationary phase, the productive time of individual microbes could be increased and the production of unwanted microbial by-products reduced, increasing process efficiency. The maximum fermentation rate could be maintained for longer periods of time. Unfortunately, the stationary phase is not well understood and not all organisms continue to produce hydrogen in this phase. Further, there is a need to distinguish between growth and making energy for the cell (i.e. for repair) and minimize reductant flux to cell precursors. It was also stated that in the continuous culture, you can either remove the cells or leave in the bioreactor, as new cells are always needed and active cells produce better, so there is potential for the issue to take care of itself without needing to produce in the stationary phase.

The next topic during the discussion was trackable target metrics for different pathways. For fermentation, the molar yield by a single organism was suggested as a trackable, relevant metric. The yield is currently limited to 4 mol H_2/mol glucose using known metabolic pathways, but may be increased to 6 or 8 mol H_2/mol glucose through reengineering of metabolic pathways. The maximum limits for yields are relatively easy to define when they are based on a defined input (such as glucose). In contrast, the upper limits of rates are harder to define as they are the result of many different steps. Another metric suggested was the percent molar yield within a given time, for example 10% in 1 hour. This would take into account that processing time will have significant impact on ultimate hydrogen cost. It was also stated that for TEA, assumptions will be needed for feedstock residence time.

It was also stated that given the current upper limit of hydrogen yield from fermentation, one alternative pathway would be develop an integrated system where the fermentation effluent was used as the feedstock for an MxC or photofermentation system. In that case, the fermentation step might not have a primary goal of hydrogen production, but instead considered as a pretreatment for the MxC or photofermentation processes, if that would make the integrated system have a higher net yield or be more economically feasible.. The goal for the fermentation step might then be acetate production or other products that could be used by a second system. If fermentation were to be considered as a pretreatment process, then TEA would need to consider both the fermentation system and the secondary system (e.g., MxC or photofermentation system) in analyses. For example, the costs for scaling–up MECs would need to be considered in terms of cost/yield trade-off.

Metrics for MECs discussed included the conversion of the precursor to hydrogen, the electrical efficiency, and the Q factor (a measure of hydrogen production generally reported in volume of hydrogen per volume of reactor anode chamber per unit of time). These metrics would allow for comparisons between systems.

Although this was the “non-light driven” day of the workshop, some thoughts were provided on metrics for photobiological systems. The light conversion, i.e. photons needed per H_2 was discussed as a key metric. One potential issue with this metric is that the conversion ratio can be manipulated by using low light levels (that is, below the level of light saturation, which is well below full sunlight for most organisms), or using only certain wavelengths. This challenge can be addressed by basing the metric on the solar spectrum, as is done by the DOE in the MYRD&D (http://www1.eere.energy.gov/hydrogenandfuelcells/mypp/pdfs/production.pdf). Photobiological hydrogen production rates were also mentioned, but this was not discussed in detail.

The discussion shifted gears again to common themes found across the breakout sessions. The first topic discussed was the need for genetic tools which was discussed in five of the six sessions across both days of the workshop. The question posed was "Where does this fit in the DOE?" since the need for genetic tools is not specific to hydrogen. It was recognized that there are limited efforts supported by the DOE Bioenergy Technologies Office (BETO) and the DOE Office of Science. The discussion focused on the need for a user facility or service provider in this area, similar to the Joint Genome Institute (JGI) and the Environmental Molecular Sciences Laboratory (EMSL) for other areas of research. Genetic tools are difficult, because the developer focuses on *their* organism and there is the issue of having the tools in the public domain versus having them proprietary. Workshop participants suggested that a facility focused on developing genetic tools and making them freely available to the public would be beneficial. It was noted that, for hydrogen in particular, there is a lack of tools compared to those for fermentation of alcohol and carbohydrates. It was also suggested that finding and studying new organisms may be faster than developing new tools.

The way in which tools are developed was also discussed as an area "ripe for change". As the most common way is to try different things until something works, a more directed path is needed. A systematic, rational approach rather than an empirical approach is needed.

The argument was made that a high throughput screening specific to hydrogen production is needed in addition to prototype development to identify weakest points in the system. These should be done in parallel with building tools and gaining a better understanding of the fundamentals of the system.

Lastly, MECs were addressed. Surface modification to improve biofilms and cellulose degradation was discussed. It was suggested that it might be helpful to immobilize cellulose-degrading bacteria on the electrode; however, this has been considered already and when the bacteria die, there are problems with regeneration. Further, the cellulose degradation pathway itself doesn't generate electrons to interact with the anode and the anode would restrict access to the cellulose. The scaling factor of MECs was discussed again with the need to reduce the cost before building a bigger system recognized as an issue. Currently the typical scale is a 1 liter system and the biggest was said to be 1kiloliter. The major performance issue between the milliliter scale and a 10 liter scale is that the proton transfer pathway is longer at 10 liter scale. A clever design could overcome this issue. The other major barriers for MECs identified were the cathode potential drop (as was found in the breakout session), the cost of high performing materials, and intellectual property issues.

Conclusions and Next Steps

Across all the workshop topics, the need for tools and broad data sets/knowledge bases was a repeated theme. Tools specifically mentioned were: genetic manipulation methods, methods to select for hydrogen production, synthetic biology tools, and *in-situ* diagnostics. Additionally, more complete information on topics ranging from: metabolic flux analysis modeling, enzyme characteristics to pathway analysis. Though these tools and knowledge bases exist for many microbial systems, tools for most species that show promise for hydrogen production are limited or non-existent, and the understanding of the energy and metabolic pathways involved with hydrogen production are not well understood. The development of tools for these species is limited at least in part by the lack of systematic genetic tool development methods.

With access to these tools and knowledge bases, researchers would be better equipped to study the energy and metabolic fluxes and regulatory systems that lead to hydrogen production. Understanding of these pathways is needed across relevant growth conditions, for example in stationary and log growth phases, and across the day/night light cycle. With that understanding, and the ability to genetically manipulate the relevant species, researchers would be able to re-direct cellular activities toward improved hydrogen production, and to evaluate the results.

To accelerate the translation of the research from the basic to applied realm, hypothesis- and target-driven research is essential. The ability to select for hydrogen production and related characteristics will greatly enhance the use of bioprospecting, random mutagenesis, combinatorial, and other screening methods that take advantage of large scale, highly diverse samples.

Integration is another theme that came up across the different workshop topics. In the near-term this generally is more focused on combining components, such as integrating multiple gene improvements into a single organism, rather than full system development. Integration allows demonstration that hypothesized interactions work as expected, and can identify issues that are not apparent when looking at components in isolation. This is particularly important for issues that are affected by the interaction of multiple components or system design, such as contamination, which though a potential problem for all biological production methods can be significantly limited by reactor design. Stability, durability, and scale-up are other issues that are affected by the emergent properties of the reactor system. For the microbial component of the system, integration may involve the genetic modification of a single species or the co-culturing of multiple species.

Though the integration of different production methods offers potential benefits, the design will require careful analysis, and TEA of the entire system will be required to identify the optimal pathways. For example, if using the organic acids produced from the fermentation of biomass to feed a photofermentation reactor, it is possible that the fermentation step should be optimized for organic acid production to get the highest net hydrogen production from the complete system.

As the systems components are integrated, it is critical that researchers in different fields and disciplines, for example, microbiologists and engineers, communicate with one another to ensure that the integration is effective, and that all relevant issues are addressed.

In addition to the technical benefits of building integrated systems, successful demonstration of integrated systems, even if not at full scale, can act as a proof-of-principle and gain support from stakeholders both inside and outside the research community.

The importance of TEA and the identification of relevant metrics was a common theme, with most of the breakout sessions specifically identifying them important as well as acknowledging the need to identify and minimize top cost contributors. Identifying relevant, measurable metrics is important both for building and evaluating TEA models, and to allow comparison of different systems across the biological hydrogen field and experimental results between different labs. Though cost is ultimately a metric of interest, it cannot be measured until biological hydrogen production systems reach an early commercialization stage where hydrogen is actually being sold. In the meantime it will be important to identify the system characteristics that can be used to evaluate progress and predict production costs and potential barriers. Rates, yields and conversion efficiencies are all possibly useful metrics. The most relevant metrics for each pathway may be different, for example solar-to-hydrogen efficiency is as irrelevant to a fermentation system as molar yield per glucose would be to a purely photolytic system, but it may be possible to identify high-level metrics that can be used to generally evaluate related pathways.

For metrics to provide a clear basis for comparison there also needs to be consensus on the conditions and measurement systems used – for example, light-based efficiencies can be dramatically affected by using low levels of light or only certain wavelengths; for this reason the FCTO has included in the definition of the solar-to-hydrogen conversion efficiency that the solar energy input is expected to be equivalent to full spectrum sunlight. To be relevant, measurable and accepted by the community, these metrics must be developed and defined by researchers working together. The DOE Photoelectrochemical Hydrogen Production Working Group provides a model for the development of such metrics and standards. The working group, consisting of researchers funded by the DOE across several different labs, has not only identified critical metrics but also published on best practices and research standards development (see http://www1.eere.energy.gov/hydrogenandfuelcells/photoelectrochemical_group.html), providing resources for the field at large. A similarly structured effort for biological hydrogen production would move the field towards a more applied focus.

Based on the results of this workshop, for the biological hydrogen production field in general, near-term research needs to include establishment of the necessary knowledge base and tools for the development of biological hydrogen production pathways. For individual pathways (e.g., photolytic, fermentation), there are more specific research needs that are addressed in the breakout sessions and final discussion sections of this report. Integration will need to occur throughout the near- to long-term, as appropriate, based on the level of technology development in a given pathway. TEA and the identification and use of key system metrics will be critical in identifying and supporting successful biological hydrogen production pathways.

Appendix A: Abbreviations and Acronyms

ANL	Argonne National Laboratory
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
BETO	Bioenergy Technologies Office
BOD	Biochemical Oxygen Demand
CBP	Consolidated BioProcessing
DOE	Department of Energy
FCTO	Fuel Cell Technologies Office
LCA	Life Cycle Analysis
MEC	Microbial Electrolysis Cell
MFC	Microbial Fuel Cell
MxC	Microbial Fuel Cell-based technology
MYRD&D	Multi Year Research, Development and Demonstration plan
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NREL	National Renewable Energy Laboratory
OPP	Oxidative Pentose-Phosphate (pathway)
PCC	Pasture Culture Collection
PSI	Photosystem-I
PSII	Photosystem-II
R&D	Research and Development
SAR	Structure activity relationship
STH	Solar-To-Hydrogen (efficiency measurement)
TCA	Tricarboxylic Acid Cycle
TEA	Technoeconomic Analysis

Appendix B: Breakout Session Tables

The full tables of issues, barriers and R&D activities generated during the breakout sessions are presented here.

Each breakout session was asked to generate answers to the same four questions:

- What issues need to be addressed to develop low-cost biological hydrogen production methods?
- What are the major barriers to developing low-cost biological hydrogen production?
- What R&D activities are needed to achieve efficient, low-cost biological hydrogen production, in the near-(now-2020), mid-(2020-2025), and long-term (2025 and later)?
- What are the key near-term activities for impact on production issues and barriers?

Participants were given stickers to use to vote on topics as follows:

- Which of the **barriers**, if resolved, would have the biggest impact on enabling low cost biological hydrogen production?
  - Participants could vote for up to five different topics each. For topics that received votes, the total number of votes are listed in the tables in blue.
- Which of these **R&D** activities would have the biggest impact on enabling low cost biological hydrogen production?
  - Participants could vote for up to five different topics each. For topics that received votes, the total number of votes are listed in the tables in green.
- What is the top R&D need in the **near-term**?
  - Participants could vote for one near-term topic each. For topics that received votes, the total number of votes are listed in the tables in red.

For the "pick your top five" voting rounds, individual participants could not vote more than once for the same card. The barriers and R&D topics that received votes are found in tables in the breakout discussion sections of this report. These tables have been edited slightly to clarify some of the topics. The full tables generated by the breakout groups can be found in Appendix B, with minimal editing beyond correcting spelling and clarifying abbreviations.

Photolytic Hydrogen Production

Table 12. **Complete Table of Photolytic Hydrogen Production Issues**
• Oxygen tolerance • Separation of H_2 and O_2 • Competing pathways • Alternative pathways to accept electrons • Compete for competitive sinks • Low cost bioreactor design • NiFe – accumulation of glycogen and increase rate • Stronger reductant or more unidirection of H_2 • Acetogenic limit – need to overcome • Alternative enzymes – use of metagenomics (O_2 tolerance) • H_2 capture and Storage – major issue • Capture H_2 from large surfaces – major issue • Down-regulation • Loading reductant into hydrogenase and/or nitrogenase • Turn-over of (glycogen) carbon source / electron source • Co-culturing for oxygen usage • Presence of inhibitory compounds

- Bubbler system
- Maximizing value of biomass produced
- Two-stage glycogen / hydrogen storage
- Breaking down glycogen
  - H_2 produced is limited due to metabolism - acetate
- Catabolic rate [of glycogen] is painfully slow
- Acetate-polymerize it to fatty acids
- Altering regulation

Table 13. Complete Table of Photolytic Hydrogen Production Barriers

- Directing electron flux to H_2 production (5)
- Photosynthetic carbohydrate storage and conversion to H_2 (4)
  - Accumulate more glycogen and starch; together with accelerating catabolic breakdown to match diurnal cycle
- Oxygen sensitivity (4)
- Carbohydrate conversion (3)
  - Increase yield of carbohydrate catabolism to make CO_2 and H_2 by overcoming the acetogenic fermentative barrier
- Linking new (O_2 tolerant) enzymes to photosystem *in vivo* (3)
- H_2/O_2 separation (3)
- Enzyme inhibition (2)
- H_2 capture and storage (2)
- Down-regulation of H2 production by non-dissipated proton gradient (2)
- Competing pathways (2)
- Maximizing reductant partitioning to hydrogenase / nitrogenase (1)
- Alternative electron sinks (1)
- Being able to capture H_2 from large light-driven surface areas – gas separation
- Coupling increased electrons to hydrogen producing catalyst instead of alternate pathways – more diverse electron donors.

Table 14. Complete Table of Photolytic Hydrogen Production R&D	
Near-term	• Development and application of metabolic engineering and synthetic biology approaches for manipulation / optimization of reductant flux to hydrogen (3) • Integration and identification of further barriers (4, 2) • Improve electron flow from water splitting via directed evolution (1, 2) • More effectively move electrons to hydrogenase, mutants with competing pathways blocked, subunits for NADH, NADPH, ferredoxin donation (2) • *In vivo* hydrogen [production] selected after mutagenesis / directed evolution (3) • Accumulate glycogen / starch to convert to CO_2 and H_2 100% (1) • Demonstrate light-driven 8 hour hydrogen production
Mid-term	• Hydrogen storage and hydrogen milking (3) ○ Pull the unfavorable equilibriums to H_2 • Repurpose existing compartments for H_2 production (3) • Engineering biological systems for high efficiency-productivity of H_2 production (2) • Demonstrate H_2 production in non-growth culture (1) • Integration of solutions (1) • Directed evolution based on phenotypic screenings • Compartmentalize H_2 production artificial hydrogenosome

Long-term	• System integration: biology and engineering (5)
	• Design/construct “synthetic hydrogenosome” *in vivo* (2)
	• Gas separation (2)
	• Comparative analysis / create hybrid systems (1)
	• Scale up and feasibility demos
	• Solar spectrum shifting to pigment spectrum
	  ○ Cheap LEDs?
	  ○ Upconversion?
	• Pathways - PPP or TCA - that generate maximum H_2 conversion, optimize carbohydrate utilization

Photofermentative Hydrogen Production

Table 15. Complete Table of Photofermentative Hydrogen Production Issues
• Definition - Electron source is not strictly water • Provision of low cost of organic/electron substrate (for PNS) • Utilization of waste streams • Politics of CO_2/sustainability/GHG • Light availability • Conversion efficiency • Using genetically modified organisms • Harvesting H_2 • Contamination

Table 16. Complete Table of Photofermentative Hydrogen Production Barriers
• Preventing contamination – i.e. H_2 consumption by other organisms (7) • Biorefinery integration (5) • How to utilize light/dark cycles (5) • Light utilization (5) • Light availability/reactor design (4) • Conversion of electrons from one form to another (3) • Nitrogenase competition of N_2 + H^+ (2) • Understanding how H_2 metabolism integrates with aerobic metabolism (1) • Preventing reoxidation of H_2 • Robustness of pure cultures

Table 17. Complete Table of Photofermentative Hydrogen Production R&D	
Near-term	• Making photosystems more efficient (5, 3) • Fundamental understanding of light/dark cycles (4, 2) • Define applied metrics, integration with experiments (1)
Near/Mid-term	• Metabolic modeling (4) • Bioprospecting and genetic tools (2)
Mid-term	• Ways to integrate biorefineries (6,1) • Reactor design (4) • Move technology to applied scale (3) • Methods to prevent contamination (2) • Increase rate of hydrogen production, i.e. electrons to nitrogenase (2) • Regulating Networks (1)

Biohybrid Systems and Enzyme Engineering Hydrogen Production

Table 18. Complete Table of Biohybrid Systems and Enzyme Engineering Issues
• Donor side turn over • Stability: coupled to water splitting (PSII or inorganic type) • Scaling – cost of materials (enzymes) • Coupling of ET - (1) proton coupling from reducing side, (2) fast back recombination • Catalyst (especially multisubunit enzymes) • Self-repair/self-regeneration • Directed assembly/delivery of enzymes • Enzyme engineering • Solar efficiency • Learn from PV – Best of industry practice • Solvent incompatibility, especially for water splitting

Table 19. Complete Table of Biohybrid Systems and Enzyme Engineering Barriers

• System integration (design rules) (8)
  ○ Limited knowledge of options to integrate components in electron transfer
• Enzymes – identification of structural determinants of catalytic efficiency and stability (7)
  ○ Need to understand the basic mechanism of the photochemical module ET -> PCET at H_2 metal catalyst site; to elucidate fundamental design principles -> make improved/new systems
  ○ No robust knowledge base for enzyme engineering
  ○ Lack of cost-effective biocatalyst production
  ○ Lack of cost-effective regeneration
• Material cost – raw material cost, manufacturing/scalability (7)
  ○ Synthetic enzymes
  ○ Cheap, robust, massively scalable cell-free translation systems
  ○ Scaling enzyme cofactor production in a low-cost manner
  ○ Low-cost enzyme/photosystem production cofactor costs
  ○ Novel materials that mimic cell membranes for enzyme stability/activity
  ○ High cost of bio-derived materials
  ○ Limited half-life of bio-derived materials
  ○ Sustainable self-regeneration with reasonable cost
  ○ Useful scaffolds to stabilize molecular entities - enable integration and systematic understanding
  ○ Immortal enzymes/catalysts
  ○ Soft-matter, self-healing supramolecular scaffolds for hybrids
• Lack of TEA/LCA comparison for bio-hybrid systems (5)
• Lack of *in situ* diagnostics at the molecular scale for bio-hybrids (5)
  ○ Lack of computational design tools for hybrid systems
• Conversion efficiency (4)
  ○ Ineffective coupling between components
  ○ Integrating the "pieces" to limit back reactions. Long-term functions

Table 20. Complete Table of Biohybrid Systems and Enzyme Engineering R&D	
Near-term	• Enzyme stabilization and activity / Bioprospecting - foundation for environmental diversity of enzyme primary structure natural diversity (8, 6) • Basic ET studies for integration (4, 2) • Technoeconomic analysis to assess cost sensitivities (5) ○ Establish performance metrics • Test bed development (2) • Fundamental studies (1) • Enzyme surface design for surface (inorganic) coupling (1) • Exploring synthetic/biosynthetic processes -> enzyme (1) • Mechanisms for regeneration • Cofactor biosynthesis – memetic synthesis • Enzyme expression – increase efficiency
Mid-term	• Design rules for enzyme engineering (SAR-SAR) (5) • "Reactor" design (2) • Test bed integration (2) • Systems integration (1) • Enzyme expression/cost reduction (large scale) (1) • Rules for increasing conversion efficiency (1) • Complex enzyme complexes: structure/function rules
Long-term	• Evolving improved biological components for coupling and efficiency (2) • Enzyme production and costs. Low-cost process (scaling) (1) • Commercial deployment (1) • Synthetic enzymes • Photolytic water splitting system • Manufacturing an integrated system

Fermentative Hydrogen Production

Table 21. Complete Table of Fermentative Hydrogen Production Issues
• Public perception of hydrogen as dangerous • Cellulose degradation

Table 22. Complete Table of Fermentative Hydrogen Production Barriers
• Understanding and development of genetic systems. (8) • Reactor design. (8) • No knowledge base – systematic knowledge on fermentative H_2 production. (7) • Better understanding of biomass convertibility. (6) • Low molar yield. (4) • Lack of available and affordable feedstocks. (4) • Processing of non-cellulosic substrates. (3) • Methanogenesis.

Table 23. Complete Table of Fermentative Hydrogen Production R&D	
Near-term	• Bioreactor design, Lab to Pilot scale (3) ○ Lab (10L) to Pilot (100L) • Bioreactors – Microbial consortia and integration (3) • Better understanding of hydrogenase (4) • Bioreactor material development (1)
Near/Mid-term	• Tailor development of biomass for H_2 production (4,1) • Genetic tools/Microbial manipulation (9,6) • Bioreactor: Solving mass transfer problems • TEA/LCA for various production options/system integration options (2)
Mid-term	• Bioreactor design, Pilot to Small Scale Industrial (1) ○ Pilot (100L) to Small-Scale Industrial (1500L) • Systems Integration – Systems biology and fermentable microbes (2) • Synthetic biology (2) • Models for electron flux
Long-term	• Bioreactor design, Small Scale Industrial to Large Scale Industrial ○ Small-Scale Industrial (1500L) to Large-Scale Industrial (??L) • Systems Integration, Demonstration (4)

Hydrogen Production by MxCs

Table 24. Complete Table of MxC Hydrogen Production Issues
• Adequate proton carriers • Cost of membranes – proton conductive (low cost) • Acidophilic and thermophilic organisms for anode • Inexpensive anode • More membrane surface area • Cathode: (1) lower overpotential, (2) better H+ reduction catalyst, (3) better H+ transport to cathode • Design: Bring electrode closer • Mass transfer limits • Controlling methanogens • Control biofilm thickness • Quality of the feedstream – (1) to control film growth, (2) defined substrates, (3) alkalinity, (4) low sulfate, (5) proton carrier (cost?), (6) protein is useful • Can H_2 help with mixing?

Table 25. Complete Table of MxC Hydrogen Production Barriers

- Testing of potential microorganisms
  - Microorganisms – function at lower pH needed (6)
  - Microorganisms – function at higher temp needed (2)
  - Controlling bad side reactions (1)
  - Low-pH tolerant ARB
  - Durability/stability of microorganism
- Cathode overpotential
  - Catalyst/electrode to reduce cathode potential loss (4)
  - Low-cost cathode materials/structures (2)
  - Better cathode catalysts (1)
  - Catalysts – H_2, cheap and fast
  - Overpotential
- Proton transport
  - Slow H+ transport (4)
  - Low-cost ion exchange membrane needed (2)
  - Proton transfer to cathode (1)
- New system configuration (1)
  - Scale up (4)
  - Low anode surface area (2)
  - Control systems – concentration, flow (1)
  - Biofilm thickness
  - Anode materials
  - Better configurations
  - Reactor cell design
  - Retention time
  - Cost – electricity, material capital
- System integration with other processes
  - Fermentation targeted to MEC feed (2)
- Identify source of potential loss (2)
- Designs tailored to feedstock

Table 26. Complete Table of MxC Hydrogen Production R&D

<table>
<tr><td>Near-term</td><td>

Cathode improvements (4, 4)

Cathode to reduce overpotential, higher-surface-area cathode (4)
Find biocatalysts (e.g., hydrogenase) that work at cathodes
Development of novel, low-cost, high-surface-area cathodes

Precisely characterize performance (5, 1)

Precisely quantify each step of potential loss that leads to overpotential (2, 1)
Characterize mech. of “potential” loss (sources of overpotential) (3 green votes)
Studies on quantifying potential losses

Fermentation + MEC integration to optimize performance (4, 1)

Target fermentation to make mostly acetate
Fermentation + MEC

Standardized metrics (4, 1)
Develop scale-up designs (6)

Study effects of “stacking” on efficiency and long-term stability of cells (4)
Investigate reactor system configurations for large module sizes and high vol. productivity (2 green votes)
Do systematic system scale up
Reactor configuration for stack up

Improved proton exchange membranes (2)

Low-cost cation and anion exchange membrane (1)
Better ion-exchange membranes (1)
Membrane development (there were two identical cards listing this)
Test/optimize materials to improve (H+) conduct

Technoeconomic analysis (2)
Monitoring of control systems

</td></tr>
</table>

Mid-term	• Improved anode organisms (3)
	  ○ Development of high-temp (~60°C) low-pH (~5) biofilm (2)
	  ○ New organisms for anode-acidophiles thermophiles (1)
	  ○ Look for and characterize low-pH ARB
	• Develop improved H+ transfer systems (2)
	• Develop improved cathode catalyst (2)
	• Scale-up – longevity, cost reduction (1)
	• Methods for biofilm thickness control
	• System control (concentration, flow)

Long-term	• Durability and stability • Improve anode with microbe engineering (metabolic/system synthetic) • Microbial metabolism understanding

Genetic and Metabolic Engineering for Hydrogen Production

Table 27. Complete Table of Genetic and Metabolic Engineering Issues

- Improvement in the H_2 to Biomass/Feedstock ratio. Hydrogen molar yield should be improved, the issue being directing cellular metabolic activity toward hydrogen rather than other products.
- Nutrient costs account for at least 40% of the overall cost of hydrogen production. Engineering organisms to utilize lower-cost nutrient (acquisition and/or use) will substantially lower the cost of hydrogen production.
- Engineering stationary phase H_2-production.
- Strategies and tools development for large-scale genetic manipulation.
- Pathway systems level predictive modeling.

Table 28. Complete Table of Genetic and Metabolic Engineering Barriers

- Pathway competition limits H_2-production (7)
- Lack of rapid an systematic genetic tools / chassis development (6)
- Inability to decouple growth from H_2-production (6)
- Lack of sufficient predictive modeling (5)
- Lack of high throughput screening approaches (3)
- Low efficiency of spent biomass recycling (3)
- High water demand in large volume H_2-production (3)

Table 29. Complete Table of Genetic and Metabolic Engineering Issues R&D

Near-term	• Development of genetic tools / chassis to increase yield of H_2-production (5, 4) • Manipulate pathways to improve yield, including heterologous expression of pathways (3, 3) • Develop stationary phase H_2-production approaches (6) • Identification of co-culture consortia that will improve yield (4) • Development of high throughput screens (3) • Use metabolic flux analysis to track energy flow (2)
Mid-term	• Engineering metabolic robustness (5) • Combinatorial tools for genetically challenging organisms or co-culture consortia (3)
Long-term	• Biology and Engineering computational integration (3)

Appendix C: Agenda

Biological Hydrogen Production Workshop Agenda

Energy Systems Integration Facility, National Renewable Energy Laboratory, Golden, Colorado

September 24-25, 2013

Tuesday, September 24, 2013

Time	Agenda
9:00 am	Welcome and Introductions (Maxwell Room, B208) ▸ **Fuel Cell Technologies Office Hydrogen Production Overview**, **Sara Dillich**, *Department of Energy* ▸ **The Hydrogen Program at NREL: a Brief Overview**, **Keith Wipke and Rich Greene**, *National Renewable Energy Lab*
9:55 am	Technoeconomic Analysis, **Brian James**, *Strategic Analysis Inc.*
10:25 am	Break
	Photobiological Session
10:40 am	Photobiological Overview, **Matt Posewitz**, *Colorado School of Mines*
11:00 am	Panel Presentations and Discussion ▸ **Photosynthetic Bacteria**, **Jake McKinlay**, *Indiana University* ▸ **Enzymes**, **John Peters**, *Montana State University* ▸ **Oxygenic Phototrophs**, **Eric Hegg**, *Michigan State University* ▸ **Biohybrid/Cell-Free Systems**, **Lisa Utschig**, *Argonne National Lab*
12:20 pm	Assemble in Breakout Rooms with Lunches (Bring $12/day for lunch)
12:40 pm	Breakout Discussions
2:40 pm	Breakout Reporting
3:20 pm	Break
3:40 pm	Full Group Discussion
5:10 pm	*Rescheduled presentation:* **Cyanobacterial Fermentation**, **Charles Dismukes**, *Rutgers University*
5:20 pm	Adjourn

Wednesday, September 25, 2013

Time	Agenda
	Non-Light Driven Biological Hydrogen Session
8:00 am	Assemble (Maxwell Room, B208)
8:10 am	Non-Light Driven Biological Hydrogen Overview, **Bruce Rittmann**, Arizona State University
8:20 am	Panel Presentations and Discussion ▸ **Microbial Fuel Cell-Related Systems**, **Jason Ren**, *University of Colorado Boulder* ▸ **Metabolic Pathways and Genetic Engineering**, **Adam Guss**, *Oak Ridge National Lab* ▸ **Bacterial Fermentative Hydrogen Production**, **Melanie Mormile**, *Missouri Science & Technology* ▸ *Presented Tuesday:* ***Cyanobacterial Fermentation***, ***Charles Dismukes***, *Rutgers University*
9:50 am	Break
10:10 am	Breakout Discussions
12:00 pm	Assemble in Main Discussion Room with Lunches (Bring $12/day for lunch)
12:20 pm	Breakout Reporting
12:50 pm	Full Group Discussion
2:20 pm	Adjourn

Appendix D: Participant List

Name	Organization
Alexander Beliaev	Pacific Northwest National Laboratory
Mary Biddy	National Renewable Energy Laboratory
Huyen Dinh	National Renewable Energy Laboratory
Sara Dillich	Fuel Cell Technologies Office, DOE
Charles Dismukes	Rutgers University
Maria Ghirardi	National Renewable Energy Laboratory
Adam Guss	Oak Ridge National Laboratory
Eric Hegg	Michigan State University
Paul Hoeprich	Nzyme2HC and Lawrence Livermore National Laboratory
Brian James	Strategic Analysis, Inc
Paul King	National Renewable Energy Laboratory
Pin-Ching Maness	National Renewable Energy Laboratory
Sergei Markov	Austin Peay State University
Jake McKinlay	Indiana University
Tasios Melis	University of California, Berkeley
Melanie R. Mormile	Missouri University of Science and Technology
John Peters	Montana State University
Matt Posewitz	Colorado School of Mines
Katie Randolph	Fuel Cell Technologies Office, DOE
Zhiyong (Jason) Ren	University of Colorado Boulder
Bruce Rittmann	Arizona State University
Louis Sherman	Purdue University
Blake Simmons	Sandia National Laboratory
Christy Sterner	Bioenergy Technologies Office, DOE
Sarah Studer	EERE Fellow, DOE
Jim Swartz	Stanford University
Lisa Utschig	Argonne National Laboratory
Philip D. Weyman	J. Craig Venter Institute
Jianping Yu	National Renewable Energy Laboratory

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